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IMPACTO DEL USO DE BIOESTIMULANTES EN LA REGENERACIÓN DE *VITIS VINIFERA L.* (cv. TEMPRANILLO)

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Analítica **Departamento de Química**

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Esta Tesis Doctoral se ha realizado en el programa de Doctorado en Análisis Forense de la Universidad del País Vasco (UPV/EHU)

Cristina E. Olavarrieta Anguiano

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Esta tesis ha sido realizada en el departamento de Química Analítica de la Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU) y en el Servicio Central de Análisis de Álava (SGIker) y consecuentemente será defendida con el propósito de obtener el título de Doctor.

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“Los obstáculos no bloquean el camino, son el camino”

Albert Einstein

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I. TABLAS

II. FIGURAS

LISTA DE ACRÓNIMOS

A	Instantaneous net photosynthesis assimilation rate
AAA	2-aminoadipic acid
AABA	α-aminobutyric acid
AACP	Products Containing Amino Acids
AAs	Amino Acids
ABA	Abscisic Acid
Acet	Acetic Acid
a.C.	Before Christ
AcOrg	Organic Acids
AcOrn	N-acetylornithine
ACN	Acetonitrile
AFM	Arbuscular Mycorrhizae Forming
Al	Aluminum
Ala	Alanine
A_N	Instantaneous Net Photosynthesis
AN	*Acoplhyllum nodosum*
ANOVA	Analysis of variance
AQC	6-aminoquinolyl-N-hydroxysiccinimidyl carbamate
Arg	*L*-Arginine
Asn	Asparagine
Asp	Aspactic Acid
ATP	Adenosín Triphosphate
AUC	Area under the curve
BABA	*β*-Aminobutyric acid
BB	Budbreak
BF	Basofoliar Avant Natur
BL	Bloom
BTOA	2-benzotyazole-2-oxyacetic acid
C	Blanck Control
Ca	Calcium
C_a	Concentration added
Ca^{2+}	Calcium Ion
CE	Capillary Electrophoresis
2-CEPA	2-chloroethylphosphonic acid

C_f	Fortified test samples
$CHCl_3$	Chloroform
ChlF	Chlorophyll Fluorescence
CH_4	Methane
C_i	Internal foliar CO_2 concentration in the substomatic cavities
Cis	*γ*-cystine
Cit	Citric Acid
Citr	Citrulline
CKs	Citoquininas
Co	Cobalt
CO_2	Carbon Dioxide
Col-0	Columbia-0 accesion
CPPU	Forclorfenuron
CRM	Certified Reference Material
C_u	Unfortified test samples
Cys	Cystenine
C16:0	Methyl palmitate
C16:1	Methyl palmitoleate
C18:0	Methyl stearate
C18:1	cis-9-oleic acid methyl ester
C18:2	Methyl linoleate
C20:0	Methyl arachidate
C20:1	Methyl Gondoic acid
C20:2	*cis*-11,14-eicosadienoic acid methyl ester
C21:0	Methyl heneicosanoate
C22:1	Methyl erucate
C4	4 Carbons
DABS-Cl	Cloruro de Dabsilo
DAD	Diode Array Detector
DAS	Days after stratification
Dns-Cl	Dansyl Chloride
DO	Denominación de Origen
DOCa	Denominación de Origen Calificada
DOP	Denominación de Origen Protegida
DW	Dry Weight

DWt32	Dry Weight at 32 DAS
DWt39	Dry Weight at 39 DAS
E	Instant Foliar Transpiration Rate
EBIC	European Biostimulants Industry Council
EI	Electronic Impact Ionization
ELSD	Evaporative Light Scattering Detector
Epi-Br	2,4-epibrasinolide
ET	Ethylene
ET_0	Reference Crop evapotranspiration
ETc	Effective Crop Evapotranspiration
EtOH	Ethanol
ETP	Evapotranspiration
eV	Electron Volt
Eq.	Equation
FA	Formic acid
FAMEs	Fatty acids methyl esters
FC	Full soil-water holding capacity
FDNB	1-Fluoride-2,4-dinitrobenzene
Fe	Iron
FIA	Flow Injection Analysis
FID	Flame Ionization Detector
Fig.	Figure/Figura
FLD	Fluorescence Detector
Fm	Maximum fluorescence in the dark-adapted state
Fm'	Maximum fluorescence in the light-adapted state
FMOC	9-fluorenylmethyl chloroformate
Fo	Minimum level of fluorescence image
Fo'	Light-adapted initial fluorescence
Fp	Peak rise in fluorescence
Fruc	Fructose
Fs	Steady state-fluorescence yield
Ft	Steady-state fluorescence in the light-adapted state
FTemp	Average foliar leaf temperature
Fv	Variable fluorescence in dark-adapted state
Fv/Fm_Lss	PSII quantum yield of light adapted sample at steady-state

FW	Fresh Weight
FWt32	Fresh Weight at 32 DAS
FWt39	Fresh Weight at 39 DAS
GA	Gibberellins
GABA	γ-Aminobutyric acid
GABA IS	γ-aminobutyric acid-2,2,3,3,4,4-d_6 Internal Standard
GC	Gas Chromatography
GC-MS	Gas chromatography coupled to mass spectrometry
Glu	L-Glutamic acid
Glu IS	Glutamine-2,3,3,4,4-d_5 Internal Standard
Gluc	Glucose
Gln	L-Glutamine
Gln IS	Glutamic acid-2,3,3,4,4-d_5 Internal Standard
Gly	Glycine
gs	Stomal conductance to water vapor
GT	Greetnal
Ha	Hectares
HCl	Hydrocloric acid
HCP	Products Containing Hormones
HDS	Honest Significant Differences
HILIC	Hydrophilic Interaction
His	Histidine
hL	Hectoliters
HomoArg	*L*-Homoarginine
HPLC	High-Performance Liquid Chromatography
HPLC-DAD	High-Performance Liquid Chromatography coupled to Diode Array Detector
HPLC-FLD	High-Performance Liquid Chromatography coupled to Fluoride Detector
HPLMC	High-Performance Liquid Magneto-Chromatography
HPLC-MS/MS	High-Performance Liquid Chromatography coupled to tandem Mass Spectrometry
Hpr	Hydroxyproline
HS	Humic Substances
HTP	Non-destructive High-Performance Phenotyping
H_2O_2	Hydrogen Peroxide
H_3PO_4	Phosphoric acid
ICH	International Council for Harmonization

i.e.	Id est
IEC	Ion Exchange
IGP	Protected Geographical Indication
Ile	Isoleucine
IPCC	Intergovernmental Panel on Climate Change
IR	Infrared
IS	Internal standard
ISO	International Organization for Standarization
K	Potassium
k^+	Potassium Ion
Kg	Kilograms
LC	Liquid Chromatography
LC-MS	High-performance Liquid Chromatography coupled to Mass Spectrometry
LED	Light-Emitting Diode
Leu	Leucine
Leu IS	Leucine-2,3,3,4,5,5,5,5′,5′,5′-d_{10} Internal Standard
LOQs	Limits of Quantification
LWC	Leaf Water Content
Lys	Lysine
L1/L2/L3/L4	Actinic illuminations
M	Maturity
Mal	Malic Acid
Malt	Maltose
ME	Matrix Effect
MeOH	Methanol
Met	Methionine
Mg	Magnesium
MG	Genetic Manipulation
mha	Thousand of Hectares
mMill.	Thousand Millions
Mill.Ha	Millions of Hectares
Mill. hL	Millions of Hectoliters
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MUFA	Monosaturated Fatty Acids

MS/MS	Tandem Mass Spectrometry
mT	milli Tesla
MTHTSM	Multi-Trait High-Performance Screening
N	Nitrogen
Na	Sodium
NAA	2-naphthaleneacetic acid
NaCl	Sodium Chloride
NADPH	Nicotiamide-Adenine Dinucleotide phosphate
$Na_2B_4O_7.10H_2O$	Sodium Tetra-Borate Decahydrate
Na_2HPO_4	Sodium Monophosphate
$NaH_2PO_4{\cdot}H_2O$	Sodium Biphosphate Monohydrate
NaN_3	Sodium Azide
NaOMe	Sodium Metoxide
NIR	Infrared Thermal Imaging
NO	Nitric Oxide
NP	Normal Phase
NPQ_Lss	Steady-state non-photochemical quenching
N_2O	Nitrous Oxide
OIVV	International Organization of Vine and Wine
OPA	3-mercaptopropionic acid
Orn	Ornithine
Oxal	Oxalic Acid
P	Phosphorus
P.	Precipitation
PAs	Polyamines
PAM	Pulse Amplitude Modilated
PAR	Photosynthetically Active Radiation
PBC	Plant Biostimulant Characterization Index
PCA	Principal component analysis
PD	Progressive drought
PGPB	Plant Growth-Promoting bacter
PGPF	Plant Growth-Promoting Fungi
PGPR	Plant Growth-Promoting Rhizobacter
PGR	Plant Growth Regulators
PH	Protein Hidrolysates

Phe	Phenylalanine
PLS-DA	Partial Least Squares Discriminant Analysis
PNUMA	Organización Meteorológica Mundial y el Programa Ambiental de Naciones Unidas
PM	Dead Plants
Pro	Proline
Pro IS	Proline-$^{13}C_5$,^{15}N Internal Standard
PSII	Photosystem II
PTFE	Polytetrafluoroethylene
PTIC	Phenylisothiocyanate
Put	Putrescine
PV	Survival Plants
PVDF	Polyvintlidenefluoride
QC	Quality Control Samples
qN_Lss	Coefficient of non-photochemical quenching in steady-state
qP_Lss	Photochemical quenching coefficient that estimates the fraction of open PSII reaction centers
QTL	Quantitative Trait Loci
QY_Lss	Steady-state PSII quantum yield
QY_max	Maximum photosystem II (PSII) quantum yield
QY_maxAUC	Area under maximum PSII quantum yield curve.
$\bar{r}$	Arithmetic means of the residues
$\|\bar{r}\|$	Average residue
R_a	Extraterrestial Solar Radiation
SFA	Saturated Fatty Acids
Rfd_Lss	Fluorescence decline ratio in steady-state
RGB	Red Green Blue Imaging
RGR	Relative Growth Rate
RH	Relative Humidity
RID	Refractive Index Detector
RMN	Nuclear Magnetic Resonance
RMS	Rotational Mass Symmetry
ROS	Reactive Oxygen Species
RP	Reverse Phase
RSD	Relative Standard Deviation
RWC	Relative Water Content

RWCt27	Relative Water Content at 27 DAS
RWCt32	Relative Water Content at 32 DAS
RWCt39	Relative Water Content at 39 DAS
SA	Salicylic Acid
Sac	Sucrose
SAH	S-AdenosinHomocysteine
SAR	Acquired Systemic Resistance
SE	Standard Error
Se	Selenium
SEC	Molecular Exclusion
Ser	Serine
SFA	Saturated Fatty Acids
Si	Silicon
S/N	Signal to-noise ratio
SOL	Slenderness of Leaves
Spm	Spermine
Spd	Spermidine
ST	SoilExpert
Succ	Succinic Acid
SV	Survival
SWE	Seaweed Extract
TAG	Triacylglycerols
Tart	Tartaric Acid
T_{min}	Minimum Leaf Temperature
TCA	Tricarboxylic Acid Cycle
TDZ	Thidiazurón
TF	Collection after harvest
Thr	Threnonine
Thr IS	Threonine-$^{13}C_4$,^{15}N,2,3,4,4,4-d_5 Internal Standard
TLC	Thin Layer Chromatography
T_{avg}	Average Leaf Temperature
T_{Max}	Maximum Leaf Temperature
TOF-MS	Time of Flight coupled to Mass Spectrometry
Tot_AAs	Total Amino Acids content
Tot_AcAAS	Total Acetyl-Amino Acids content

Tot_Met$_{t32}$	Total Metabolites accumulation at 24 hours after the first foliar application
Tot_Met$_{t39}$	Total Metabolites accumulation at 24 hours after the second foliar application
Tot_number	Total number of seeds
Tot_Org	Total Organic acids content
Tot_weight	Total weight of seeds
Trp	Tryptophan
tSpm	Thermospermine
TV	Collection before harvest
TW	Turgid Leaf Mass
Tyr	Tyrosine
T0A	Collection before the first foliar application
T0B	Collection before the second foliar application
T24A	Collection 24 hours after the first foliar application
T24B	Collection 24 hours after the second foliar application
UE	European Union
UFA	Unsaturated Fatty Acids
UPLC-MS/MS	Ultrahigh-Performance Liquid Chromatography coupled to tandem Mass Spectrometry
UV	Ultraviolet
V	Veraison
Val	Valine
WD	Water Deficit
WUE	Water-Use Efficiency
Zn	Zinc
$\sigma(r)$	Arithmetic means of the residues
σ_{TOT}	Standard deviation of the residues
1,000_seeds	Weight of 1,000 seeds
^{14}C	Carbon-14

Resumen

Como consecuencia de los cambios climáticos ocurridos durante las últimas décadas se prevé una intensificación de las temperaturas y de los periodos de sequía en muchas regiones vitivinícola, donde *Vitis vinifera* está sufriendo desajustes en sus ciclos reproductivo y vegetativo, así como en su fisiología y metabolismo, llegando a un debilitamiento del rendimiento y la calidad de los frutos producidos. La aplicación de bioestimulantes aparece como una herramienta agronómica ecológica, innovadora y sostenible para minimizar los efectos negativos inducidos por la climatología adversa. Sin embargo, aún no está claro su mecanismo de acción en la vid.

En este estudio se evaluó el mecanismo de acción de sustancias bioestimulantes comerciales (dos a base de extractos de algas marinas Ascophyllum nodosum y un bioestimulante a base de aminoácidos vegetales), junto con un aminoácido no proteico como el ácido β-aminobutírico para comprobar cómo su uso, en campo abierto, afecta en la producción de una planta leñosa como *Vitis vinifera* (var. Tempranillo) poco productiva ubicada en La Rioja (España), durante dos temporadas, debido al escenario del cambio climático. En *Vitis vinifera* se desarrolló un plan de campo experimental definido por la aplicación foliar de tres productos bioestimulantes comerciales, junto al aminoácido no proteico BABA para determinar su mecanismo de acción a través de su perfil fisiológico y bioquímico. Se cuantificaron metabolitos como carbohidratos, ácidos orgánicos y aminoácidos en diferentes tejidos vegetales a través de cromatografía líquida de alto rendimiento (HPLC) acoplada a diferentes detectores (RID, DAD y FLD) para obtener una mayor sensibilidad y menor incertidumbre en la medida, y determinar su mecanismo de acción a través de su perfil fisiológico y bioquímico.

Además, se evaluaron diversas accesiones de una planta modelo como *Arabidopsis thaliana* cultivada bajo dos regímenes de riego (condiciones óptimas y sequía progresiva) en condiciones controladas durante ciclos de estrés hídrico a corto y a largo plazo y tratadas con los mismos productos bioestimulantes comerciales. Gracias al empleo de una planta modelo como es Arabidopsis se evaluaron los cambios morfo-fisiológicos a través de un fenotipado de alto rendimiento de rasgos múltiples (MTHTSM), cambios bioquímicos a través de la determinación de ácidos orgánicos y aminoácidos, cambios en la producción a través de la determinación de ácidos grasos mediante cromatografía de gases acoplada a espectrometría de masas, para evaluar la traducción biológica entre ambas plantas cultivadas en condiciones de déficit hídrico.

Para el análisis, se emplearon diversas herramientas estadísticas, como multi-ANOVAs y PLS-DA en el caso de *Vitis vinifera* y multi-ANOVA, PCA y matriz de correlación de Pearson en el caso de *Arabidopsis thaliana.*

La falta de agua provocó una disminución en los parámetros hídricos, observando que la simple aplicación de los productos bioestimulantes produjo una alteración en la respuesta fisiológica de ambas especies. Sin embargo, existe cierta controversia en función de la especie. Mientras que en *Vitis vinífera*, la aplicación de bioestimulantes produce una mejora en la respuesta hídrica bajo condiciones de estrés hídrico severo en *Vitis vinífera*, en *Arabidopsis thaliana*, la aplicación de bioestimulantes bajo ambientes extremos de sequía produce una intensificación del déficit hídrico que compromete el rendimiento del cultivo, mostrándose en ambos casos a través del contenido relativo/foliar de agua (LWC/RWC) y en la eficiencia del eso del agua (WUE).

Estos indicadores muestran que en *Arabidopsis thaliana* solo se promueve el crecimiento en condiciones óptimas de riego, siendo este efecto dependiente de la concentración o del tiempo de aplicación, mientras que en *Vitis vinifera*, los productos bioestimulantes son capaces de mejorar el rendimiento y la eficacia del cultivo incluso en ambientes de sequía moderada. Sin embargo, la aplicación de bioestimulantes bajo condiciones abiertas de campo sobre *Vitis vinifera* muestra una clara dependencia en función de la ubicación de las vides y de la climatología de la añada estudiada. Como bien se ha reportado la principal fuente de variabilidad radica en que, bajo condiciones controladas, las plantas crecen en macetas individuales, mientras que en campo abierto las plantas compiten con todo el dosel por los recursos hídricos, y cada planta puede presentar un genotipo diferente[449], siendo de gran valor un futuro estudio sobre cada fenotipo individual.

La aplicación de bioestimulantes juega un papel fundamental en el metabolismo primario y la respuesta al estrés hídrico, en función del principio activo empleado. Las vides rociadas con BABA acumularon un mayor contenido de aminoácidos libres, especialmente arginina y glutamina en periodos finales del ciclo vegetativo, así como presentó una mayor protección frente a plagas como el mildiu. Así mismo, los productos comerciales promueven la calidad de mostos y uvas gracias a la acumulación de ácidos orgánicos, sobre todo el producto comercial SoilExpert. Sin embargo, la acumulación de solutos activos osmóticamente en rosetas de Arabidopsis rociadas con los productos bioestimulantes además de promover el crecimiento sirve como como marcador de la tolerancia al estrés hídrico.

Nuestro estudio demostró que la aplicación de bioestimulantes puede mejorar el crecimiento de la planta, la calidad y cantidad de los cultivos, aunque la respuesta fisiológica y su perfil bioquímico depende de la combinación especie-bioestimulante.

Capítulo I: Introducción

1.1 HISTORIA Y CARACTERÍSTICAS DE LA VID

El cultivo de la vid y la elaboración del vino están asociados a la historia de la humanidad desde tiempos remotos. La primera evidencia arqueológica referida a restos vitivinícolas data del año 5400 a.C. en Oriente Medio, en las montañas de Zagros (Irán), donde se encontró una vasija, conservada en el museo de la Universidad de Pensilvania, con trazas de cristales de tartrato de calcio[1] que indicaban el almacenaje de vino en su interior. Por otro lado, el estudio mediante técnicas paleobotánicas de pepitas de uva descubiertas en Armenia (provincia de Vaytoz Dzor, frontera con Irán y Turquía) en torno al año 2500 a.C, permitió asignar dichas pepitas a la especie *Vitis vinifera*, una variedad de vid doméstica que todavía hoy en día se cultiva. Los expertos consideran que, la Viticultura nació en las montañas de Armenia, Georgia y países vecinos, como Irán.

En la cultura egipcia, el vino era considerado un lujo reservado a sacerdotes y nobles, favoreciéndose su comercialización a través del Delta del Nilo. Procedente de Egipto, la siguiente etapa de expansión de la viticultura se localiza en Creta (Grecia), donde comenzó a cultivarse la vid desde el año 700 a.C. A su vez los griegos fueron los encargados de introducir su cultivo en la cultura mediterránea[2].

En el sur de Francia, la Península Ibérica y a orillas del Ródano, los romanos impulsaron el desarrollo de la viticultura e introdujeron avances técnicos para el comercio como el ánfora, el lagar y la barrica. En España, el paisaje de viñedos se fue estableciendo a lo largo de la Edad Media y Moderna, apareciendo las plantaciones de vid en diferentes terrenos e implantándose complejos sistemas de cultivo, elaboración y comercio acorde a las prescripciones religiosas del momento, una idea inseparable hasta el siglo XII[3].

No fue hasta el año 1500 cuando la vitivinicultura llegó a México, América del Sur, y posteriormente a otros lugares como Sudáfrica (1655), como consecuencia de la expansión colonial. Así mismo, a principios del siglo XIX, los monjes mejicanos y españoles, siguiendo la costa del pacífico, expandieron el cultivo de la vid en la actual California. En Australia, los primeros pobladores europeos introdujeron la vid a finales del siglo XVIII y llegaría a Nueva Zelanda a principios del siglo XIX[2,3].

La palabra vid proviene del latín, *vitis*, que deriva del vocablo griego *bios*, "vida", aludiendo a la capacidad de regeneración de la planta tras su muerte invernal o a su capacidad de vegetar y prosperar bajo condiciones climáticas adversas[4].

Existen 60 especies de vides del género botánico *Vitis* con diversos subgéneros y orígenes. Así, por ejemplo, con el subgénero *Muscadinia* destacan *Vitis rotundifolia*, *Vitis munsoniana* y *Vitis popenoi*. En el subgénero *Euvitis*, provenientes tanto de América como de Asia o Europa destacan, *Vitis aestivalis*, *Vitis lambrusca*, *Vitis lincecumii*, *Vitis calcifornica*, *Vitis riparia*, *Vitis rupestris*, *Vitis berlandieri*, *Vitis cordifolia*, *Vitis candicans*, *Vitis cinérea*, *Vitis amurensis*, *Vitis sylvestris* y *Vitis vinifera* entre otras. De todas ellas, la especie *Vitis vinifera* L. cultivada en Europa desde la antigüedad es la que posteriormente se ha expandido a otros continentes para la producción enológica. Posee infinidad de variedades que aportan diferentes características organolépticas a los vinos y representa más del 90% de la producción a nivel mundial. Así mismo, *Vitis rupestris*, *Vitis riparia* y *Vitis berlandieri* han sido las más empleadas, entre otros usos, para la constitución de portainjertos[5].

La vid es una planta liana, leñosa y de hoja caduca con un ciclo juvenil de entre 3 y 5 años[6,7]. Desde la crisis de la filoxera (siglo XIX), se utiliza el sistema de injerto, que consiste en la unión de dos individuos. Uno de ellos, constituye el sistema radical (*Vitis spp*.) del grupo americano principalmente denominado patrón o portainjerto (inmune a la filoxera), y otro, la parte aérea (*Vitis vinifera* L.) denominada púa o variedad[6]. Esta última parte constituye el tronco, los brazos y los pámpanos, que aportan las hojas, los racimos y las yemas. La unión de ambas zonas es lo que se conoce como cepa[6,7]. En este contexto es importante mencionar que la crisis de la filoxera (1868) se produjo por la importación desde el estado de Georgia (USA) de la variedad "Isabela" de vid americana, con el objetivo de combatir la plaga de oídium que estaban sufriendo en ese momento las vides europeas. Estas importaciones introdujeron también el insecto filoxera (*Daktulosphaira vitifoliae*), que ya en Europa, fue poco a poco eliminando las vides europeas, no adaptadas a estos tipos de insectos[8].

El sistema radicular sirve como anclaje de la planta al suelo, para la absorción de agua y elementos minerales y acumulación de sustancias de reserva *(Fig. 1.1)*. Las raíces pueden proceder de la semilla (raíz principal) o de las células del periciclo (capa rizógena) que pueden aparecer en tronco, brazos o sarmientos o como raíces subterráneas[7].

La parte aérea comprende el tronco, los brazos/ramas y los brotes (pámpanos). El tronco tiene aspecto retorcido, sinuoso y agrietado, recubierto exteriormente por una corteza que se desprende en tiras longitudinales, donde se almacenan sustancias de reserva y conducen la savia y el agua. Su altura depende de la poda de formación, generalmente comprendida entre 0 y 2 m[6-7].

Los brazos son los encargados de conducir nutrientes y repartir la vegetación y los frutos en el espacio. Los brazos portan los tallos herbáceos del año, denominados pámpanos. El pámpano es un brote procedente de una yema normal y soporta las yemas, las hojas, los zarcillos y las inflorescencias[6,7,9,10]. Sin embargo, cuando comienzan a lignificarse adquieren consistencia leñosa denominada sarmiento.

Los pámpanos son tallos formados con una sucesión de nudos *(Fig. 1.1)*. Estos nudos son ensanchamientos donde se insertan los diferentes órganos[6,7,9,10]. Los diferentes órganos que portan los pámpanos y sarmientos en los nudos son varios. Uno de ellos son las hojas que se sitúan de forma alterna y opuestas, y están compuestas por peciolo y limbo. También se encuentran las yemas que son brotes de los cuales surgen los tallos con sus hojas, inflorescencias, zarcillos y nuevas yemas. De las yemas pueden obtenerse zarcillos que son tallos modificados que sirven como sujeción y le permiten a la vid la capacidad de trepar.

Por último, se encuentran las flores y el fruto, que aparecen en forma de inflorescencia, denominada racimo, y que se encuentra opuesto a las hojas. El racimo está compuesto por el pedúnculo, hombros, raquis y pedicelo. La vid suele poseer entre uno y tres racimos por pámpano fértil y al conjunto de ramificaciones del racimo se denomina raspón[6,7,9,10]. que aparecen en forma de inflorescencia denominada racimo, y que se encuentra opuesto a las hojas (*Fig. 1.1*). El racimo está compuesto por el pedúnculo, hombros, raquis y pedicelo. La vid suele poseer entre uno y tres racimos por pámpano fértil y al conjunto de ramificaciones del racimo se denomina raspón[6,7,9,10]. El pedicelo del racimo proporciona las flores, en este caso, hermafroditas y de unos 2 mm de longitud, compuestas por cáliz, corola, androceo y gineceo. El fruto es una baya de forma y tamaño variables, más o menos esférica u ovalada de unos 12-18 mm de diámetro. Las bayas están compuestas por hollejo, pepita y pulpa[6,7,9,10].

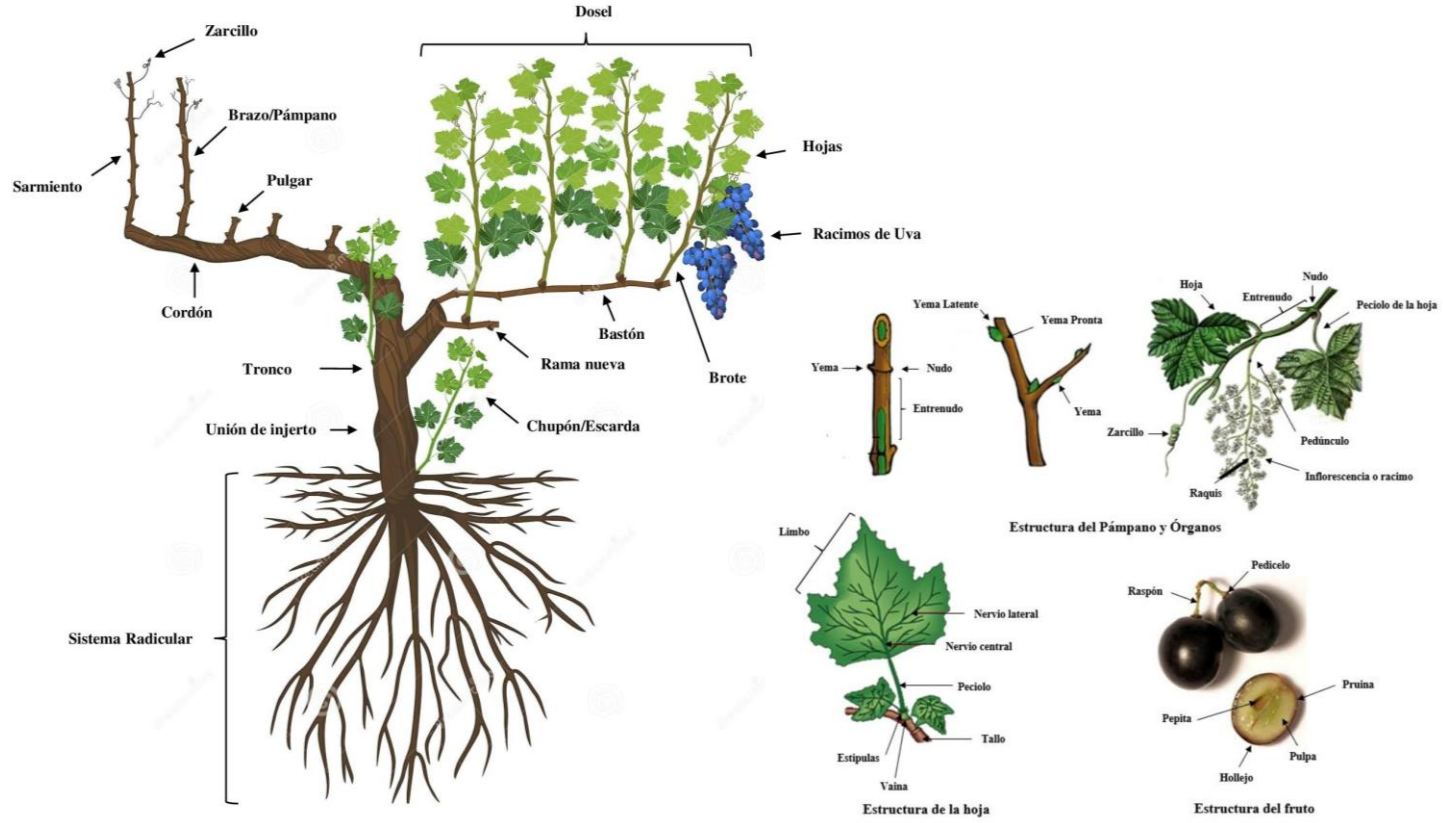


Figura 1.1. *Morfología de la vid y estructura de sus órganos, hoja y fruto. Adaptado de " https://urbinavinos.blogspot.com/2015/08/morfologia-de-la-vid-vitis-vinifera-l.html., Ihttps://dreamstime.com. y. https://hogardevinos.com/la-vid/morfologia-de-la-vid/" [7,11,12].*

1.2 IMPORTANCIA DE LA PRODUCCIÓN DE VINO EN ESPAÑA

El sector vitivinícola representa una parte importante de la actividad social, cultural y económica en muchas regiones del mundo. En un informe reciente de la Organización Internacional de la Viña y el Vino (OIVV), la superficie destinada a este cultivo se estabiliza en 2016 y se expande en 2019 hasta las 7,4 Mill. ha de las cuales 966 mha corresponden a España, siendo así el país con mayor superficie de viñedo del mundo[13]. Basándonos en dicho informe, la producción mundial de vino alcanzó en 2019 los 260 Mill. hL, de los cuales en España se producen un 12,9% del total mundial que corresponde a 33,5 Mill. hL, superado únicamente por países como Francia e Italia, en algunas añadas. En cuanto a exportaciones, España se encuentra en torno al 21,3% Mill. hL convirtiéndose así, junto a Italia, en uno de los países líderes en exportación mundial de vino[13].

La cuenca mediterránea y en particular la península Ibérica son zonas privilegiadas para la producción de vinos con características muy diferenciadas debido a su situación geográfica, diferencias climáticas y variedad de suelos. Conviven regiones muy dispares que presentan desde temperaturas elevadas y luminosidades en torno a 3000 horas/año que aceleran la maduración y producen vinos dulces, hasta zonas con temperaturas bajas y luminosidades en torno a 2000 horas/año que aportan vinos de mayor acidez. Así mismo, la pluviosidad favorece que las lluvias de invierno aporten calidad en la vendimia y la escasez de agua en verano aumente el rendimiento de producción.

Concretamente y en la actualidad, España cuenta con 97 regiones de producción de vinos de calidad con Denominación de Origen Protegida (DOP), de las cuales 70 son con Denominación de Origen (DO) y, solamente dos cuentan con la distinción de ser Denominación de Origen Calificada (DOCa), Rioja y Priorato, obtenidas en el año 1991 y 2000 respectivamente. Además, siete de las regiones cuentan con Vinos de Calidad con Indicación Geográfica (IGP) y 26 son Vinos de Pago[14]. Todos ellos, siguen el modelo europeo de producción, manteniendo un estricto control sobre la cantidad producida, las prácticas enológicas y la calidad de los vinos de cada zona[15]. La Ley 24/2003, de 10 de Julio, de la Viña y del Vino, definen los conceptos anteriormente mencionados[16].

De toda la superficie española destinada a viñedo, Castilla-La Mancha ocupa el 48,8% de la superficie total nacional, seguido por Extremadura (8,6%), la Comunidad Valenciana y Castilla y León (7%), Cataluña (6%) y La Rioja (5%)[3].

1.3 DENOMINACIÓN DE ORIGEN CALIFICADA RIOJA (DOCa Rioja)

Los antecedentes históricos de la vid en La Rioja se remontan a la época Medieval, donde el cultivo de trigo y de vino tuvo un peso importante en la economía de la región. Poetas como Gonzalo de Berceo, que profesó como monje en el monasterio de San Millán de la Cogolla ya ensalzaban las virtudes del vino Rioja[17].

Quiero fer una prosa en román paladino
en qual suele el pueblo fablar con so vecino,
ca non so tan letrado por fer otro latino,
bien valdrá como creo, un vaso de bon vino

La transición del medievo a la edad moderna supone la consolidación del vino Rioja debido al incremento de su producción. En 1787 se creó la Real Junta de Cosecheros, cuyo objetivo era el fomento del cultivo de vid, mejorar la calidad de los vinos y facilitar su comercialización[17].

Así mismo, durante la segunda mitad del siglo XIX se comienzan a crear las que hoy en día se conocen como bodegas centenarias que, gracias a la introducción de nuevos métodos de elaboración y crianza, aportaban a los vinos perfiles bien diferenciados. Además, gracias a la llegada del ferrocarril y a la crisis de la filoxera, la comercialización de los vinos de Rioja sufrió un importante crecimiento.

Debido a la preocupación de los viticultores por proteger la identidad propia de un producto vinculado a su origen, en 1925, se reconoce oficialmente la Denominación de Origen Rioja, pero no fue hasta 1970 cuando se aprobó el primer Reglamento de la Denominación de Origen y de su Consejo Regulador, actualmente definido por la Orden APA/3465/2004, de 20 de octubre de 2004[18], donde se establecen los principios esenciales de producción, demarcando las zonas de producción, las variedades de uva autorizadas, las prácticas culturales, las normas de campaña para la regulación de la vendimia, los rendimientos máximos de producción, y todo lo relacionado con las plantaciones. También especifica las prácticas de elaboración, las características del envejecimiento, la calificación y características de los vinos, los registros necesarios, los derechos y obligaciones, así como las normas de control y vigilancia de la denominación para la calificación de los vinos.

La DOCa Rioja se considera una región privilegiada para el cultivo de vid y elaboración de vinos de alta calidad, siendo la marca "Rioja" una de las cinco regiones con mayor notoriedad entre las zonas

vitivínicolas más prestigiosas del mundo[19]. Esta región, situada en el valle del Ebro, se divide en tres zonas principales, Rioja Alta, Rioja Alavesa y Rioja Baja (*Fig. 1.2*), las cuales concentran una superficie útil de viñedo de 66 mha, con una producción, en 2019, de 2,7 Mill. hL[20].

Figura 1.2. *Regiones que comprenden la DOCa Rioja. Fuente: https://www.bodegasmontecillo.com/las-tres-zonas-produccion-la-doca-rioja/*[21].

Cada una de las zonas en las que se divide la región presenta diversas características climáticas y de composición de suelos. En la zona Occidental, compuesta por las áreas de Rioja Alta y Rioja Alavesa, predomina la influencia del clima atlántico, con inviernos largos, veranos fríos y otoños templados. Rioja Baja, por el contrario, presenta un clima más seco y cálido, con inviernos soleados y veranos más calurosos y secos debido a la influencia mediterránea[22,23]. En Rioja Alta y Rioja Alavesa, predominan los suelos arcillo-calcáreos situados en terrazas y pequeñas parcelas, que proporcionan vinos glicéricos y de elevada acidez. En Rioja Baja predominan principalmente los suelos aluviales y arcillo-ferrosos. Los suelos aluviales, próximos a los ríos, proporcionan vinos con mayor graduación alcohólica y acidez moderada. Todo ello proporciona a la denominación un ambiente adecuado para la elaboración de vinos de calidad con una personalidad e identidad reconocible dentro del abanico de posibilidades que ofrece la viticultura[22,23].

Esta tendencia climática refleja a su vez precipitaciones abundantes en torno a unos 400 mm, una condición idónea para la producción de vid y que progresivamente descienden hacia el este. En las zonas con mayor influencia oceánica, las lluvias de invierno son muy abundantes, incluso llegando a ser tan importantes como en el máximo primaveral, donde se reciben entorno al 30-35% de las precipitaciones. Los meses de verano y más concretamente Septiembre, son los meses más secos del año[23].

Esta región presenta temperaturas medias anuales inferiores a los 12°C. En los meses de Mayo y Junio presenta un ascenso acentuado de unos 4°C, marcando el paso hacia veranos templados. Aunque la región no soporta acusados excesos térmicos, los promedios de las temperaturas máximas y mínimas refuerzan la consideración de clima templado. Excepcionalmente las máximas absolutas han superado los 35°C, sobre todo en los últimos años, indicando así un contraste importante entre verano e invierno. Durante el transcurso del año, la evapotranspiración (ETP) es muy baja en invierno y demasiado alta en verano, y en cuanto al balance hídrico anual es claramente negativo con valores alrededor de -100 mm en Rioja Alta[23].

En la DOCa Rioja, las variedades vitícolas autorizadas por el reglamento son los varietales tintos Tempranillo, Garnacha, Graciano, Mazuelo y Maturana, siendo la variedad Tempranillo, autóctona de La Rioja, la variedad más característica que confiere la identidad de los vinos de esta denominación. Ocupa más del 75% de la superficie de cultivo y es muy versátil, enológicamente hablando, puesto que es capaz de producir vinos con largo envejecimiento, muy equilibrados en grado alcohólico, color y acidez, y con un paladar suave y afrutado que evoluciona a aterciopelado cuando envejece. Las variedades blancas son Viura, Malvasía, Garnacha blanca, Tempranillo blanco, Maturana blanca, Turruntés, Chardonnay, Sauvignon Blanc y Verdejo[24].

1.4 INFLUENCIA DEL CAMBIO CLIMÁTICO EN LOS CULTIVOS

En las últimas décadas del siglo XX se registraron una serie de cambios climáticos que influyen en la producción vitivinícola y que requieren máxima prioridad para adaptar los cultivos a las nuevas condiciones atmosféricas. Principalmente se ha detectado un aumento atmosférico en la concentración de dióxido de carbono (CO_2), metano (CH_4) y óxido nitroso (N_2O)[25], éstos últimos considerados dos de los gases más potentes del efecto invernadero. Este hecho ha generado un

aumento en la temperatura terrestre y, a pesar de existir pocos estudios que evalúen la respuesta de la vid al aumento de la temperatura y CO_2, se prevé que en climas cálidos aumente la evapotranspiración[26], generando cierto déficit hídrico en la planta, sobre todo durante los meses más secos del año.

Como consecuencia del cambio climático también se han producido cambios en los regímenes de precipitación, de menor frecuencia y muy caudalosos que generan graves consecuencias sobre la viticultura y más concretamente sobre el ciclo hidrológico, a pesar de ser una planta resistente a diversos cambios ambientales[26-28]. Además del estrés directo sobre las plantas, el cambio climático podría ampliar la gama de patógenos y plagas, aumentando su frecuencia y/o la gravedad de los brotes de enfermedades, sobre todo en vid, donde se ha reportado que la exposición a factores ambientales adversos puede modificar sus mecanismos de defensa contra insectos[29,30].

Existen estudios que revelan el calentamiento promedio producido en varias regiones vitivinícolas en todo el mundo, donde se alcanzan valores de entre 1,3 y 1,7°C desde 1950 hasta 2004 y las perspectivas para los próximos años estima un calentamiento de 0,2°C por cada década, alcanzando valores de entre 1 y 6°C en el siglo XXI[31-34]. Más adelante en el apartado 1.5.1 se desglosarán algunos aspectos relacionados con este tema.

Este hecho científico contrastado y estudiado permanentemente desde 1988 por el Panel Intergubernamental en Cambio Climático (IPCC, Intergovernemental Panel on Climate Change) de la Organización Meteorológica Mundial y el Programa Ambiental de Naciones Unidas (PNUMA), no solo afecta a cultivos específicos como la vid, sino que evidentemente abarca una problemática general en términos agropecuarios. En los últimos 20 años, la exposición a los choques climáticos no solo ha aumentado en términos de frecuencia sino también de intensidad, produciendo vulnerabilidades por riesgo de inseguridad alimentaria debido al incremento de los choques climáticos[35]. Se ha estimado que en los próximos años se esperen pérdidas de entre el 30% y 50% en las producciones agrícolas mundiales debido al estrés biótico o abiótico, o ambos que están sufriendo los cultivos[36]. Estas pérdidas, junto con el aumento constante de la población humana y el uso de casi el 70% de las reservas de agua dulce en forma de riego, indican que se requiere un aumento del 60% en la producción agrícola para satisfacer las necesidades globales[27].

Para ayudar a mejorar la producción agrícola y la seguridad alimentaria, una estrategia sostenible consistiría en aumentar la resistencia y resiliencia de las plantas para contrarrestar las tensiones inducidas por el cambio climático[35,37].

1.5 COMPORTAMIENTO DE LAS PLANTAS BAJO CIERTOS FACTORES DE ESTRÉS

Los factores climáticos juegan un papel crucial en el rendimiento de las plantas, ya que las situaciones ambientales adversas pueden influir en el crecimiento, desarrollo y producción de los cultivos, pudiendo provocar una disminución de la calidad, daños permanentes o incluso la muerte[38,39]. Por este motivo, las plantas deben tolerar las condiciones ambientales adversas a través de cambios metabólicos o mecanismos reguladores de la respuesta a estrés[39].

El concepto de estrés fue desarrollado originalmente en 1936 por Hans Sely, y desde un sentido puramente biológico, lo aplicó en la descripción de las limitaciones ambientales y desfavorables de las plantas[40]. Sin embargo, en 1987, Walter Larcher lo definió como un "estado en el que las crecientes demandas que se le hacen a una planta conducen a una desestabilización inicial de funciones, seguida de una normalización y una mejor resistencia". Además, consideró que "si se superan los límites de tolerancia y se sobrecarga la capacidad de adaptación, el resultado puede ser un daño permanente o incluso la muerte"[32,40].

Según esta definición, el estrés es un estado temporal en el que la planta experimenta un cambio que exige una respuesta, donde si la demanda está dentro de los límites de tolerancia, la respuesta de la planta lo restaura al estado normal. Sin embargo, si la planta no logra lidiar adecuadamente con el estrés podría provocar su muerte[39,40]. En contraposición, Lichtenthaler en 1996, incluyó en el concepto de estrés vegetal las fases de regeneración de las plantas, cuando eliminan los factores estresantes[40].

A partir de aquí se ha clasificado el estrés vegetal en función de varios factores[39].

1) En función del factor que lo causa:

a) Biótico: como el resultado de interacciones entre la planta y otro organismo vivo que comparten el mismo medio ambiente y que resulte en daños parciales o significativos que determinen la supervivencia de la planta. Se incluyen bacterias, hongos, virus, nematodos o parásitos[41].

b) Abiótico: como el resultado de cambios en factores no biológicos, principalmente ambientales o nutricionales, como la sequía la temperatura o la salinidad, que afectan al crecimiento, la reproducción o la vida de la planta y son el principal factor que afecta a la productividad[42].

2) En función del efecto del estrés[40,41].

a) Eu-estrés: situación activadora beneficiosa en el desarrollo de la planta.
b) Dis-estrés: situación severa y negativa en la planta.

3) Según las tensiones de la planta[39]:

a) Tensiones internas: que provienen del interior de la planta.
b) Tensiones externas: que existen fuera de la planta.

A pesar de las diferencias entre las diversas definiciones de estrés vegetal, todas se centran en describir un cambio en las condiciones que afectan a la planta, la respuesta de la planta a este cambio y el nivel de daño que puede provocar el cambio. Por ello, los mecanismos de los que se ayuda la planta en situaciones de estrés se centran en 4 fases basadas en la duración y la intensidad del factor estresante (*Fig. 1.3*)[39,40,43].

- ***Fase de alarma:*** es la fase en la que la planta reacciona al estrés activando mecanismos y produciendo un cambio en sus condiciones óptimas de crecimiento como la fotosíntesis o el transporte de metabolitos. Como consecuencia se produce una desviación en las condiciones fisiológicas como una disminución de su vitalidad o una mayor tasa de catabolismo[40].

- ***Fase de resistencia/restitución:*** la planta responde a los factores estresantes que afectan a su crecimiento y reproducción mediante procesos de adaptación, reparación y endurecimiento, proporcionando un nuevo estado fisiológico frente al efecto prolongado o la excesiva dosis alcanzando un máximo de resistencia[40].

- ***Fase de agotamiento, fase final:*** cuando la planta está expuesta de forma prolongada al factor estresante o a una excesiva dosis de estrés y, los mecanismos para afrontarlo no logran una mejora, se produce pérdida progresiva de vitalidad. Si estas condiciones estresantes se prolongan, se generarán daños graves e incluso la muerte dependiendo de la especie, el tiempo y la dosis[40].

- ***Fase de regeneración:*** cuando el factor estresante se elimina, se logra una restauración parcial o total de los estándares fisiológicos y la planta podrá sobrevivir. Dependiendo del tiempo y la etapa del agotamiento donde se elimine el factor estresante, los nuevos estándares fisiológicos se encontrarán entre el mínimo y el máximo de resistencia, por lo tanto, la planta vivirá, pero con limitaciones en el crecimiento o en la capacidad de reproducción[40].

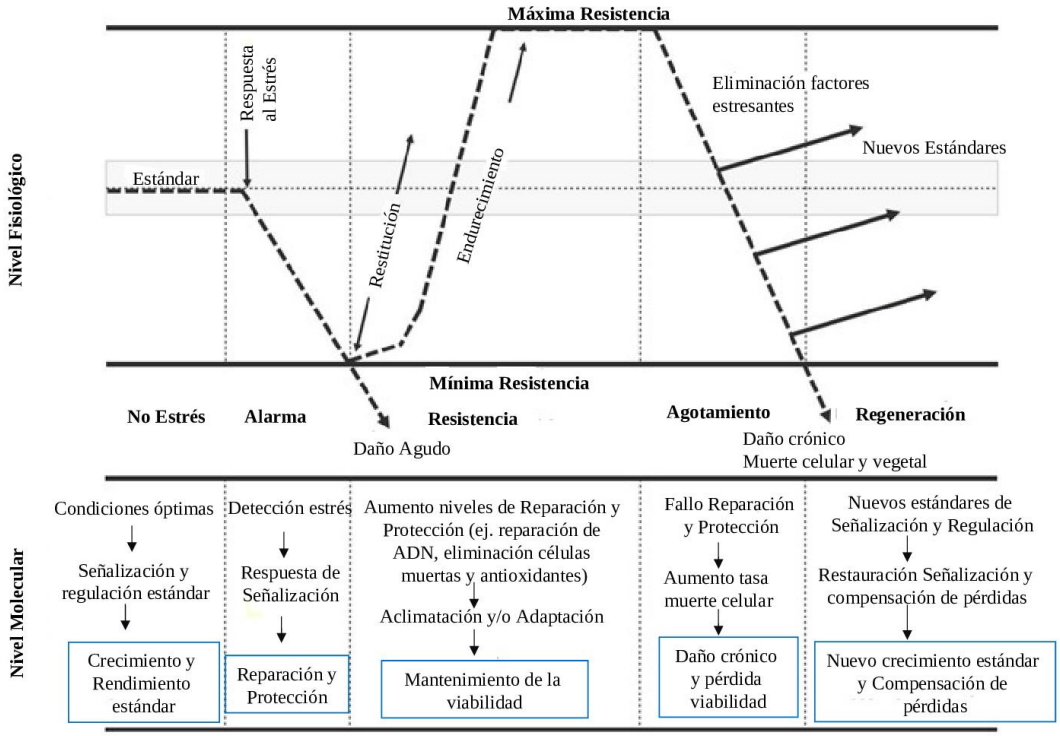


Figura 1.3. *Secuencia de fases y respuestas inducidas por exposición a un factor de estrés. Adaptado de "Mosa, K. A.; Ismail, A.; Helmy, M. Plant Stress Tolerance: an Integrated Omics Approach; Springer: Switzerland, 2017"*[39].

1.5.1 EFECTO DE LA TEMPERATURA Y LA SEQUÍA EN LOS VIÑEDOS

Uno de los objetivos principales de este trabajo es estudiar la respuesta de *Vitis vinifera* frente al daño extremo que ha sufrido durante los últimos años, debido a factores abióticos derivados previsiblemente del incipiente cambio climático que se observa en algunas zonas vitivinícolas. El estrés generado por temperaturas extremas o por estrés hídrico son dos de los factores ambientales que más negativamente afectan al crecimiento y productividad de las plantas, especialmente en regiones semiáridas como Rioja Alta[44].

En términos generales, las altas temperaturas y consecuentemente el déficit hídrico que provoca impactan en la fisiología de las plantas aumentando la transpiración de las hojas y las tasas de respiración, afectando a la fotosíntesis[34]. Se sabe que la respuesta de la planta depende de la etapa de desarrollo y del nivel de estrés al que estén sometidas las vides[45]. Por ello, a corto plazo, la

primera respuesta de la planta ante este tipo de estrés es el cierre de estomas, que consecuentemente provoca menores tasas de fotosíntesis y transpiración porque reduce la fijación de carbono en la planta y por lo tanto genera problemas en el rendimiento[46-48]. Si el estrés es prolongado, debido a la falta excesiva de CO_2 derivada del cierre estomático, se inhibe totalmente la fotosíntesis, y la falta de transpiración hace que la planta incremente su temperatura. Además, la conductancia hidráulica se ve por ello afectada y la toma de agua interrumpida, y con ello se reduce la turgencia de los tejidos limitando pasivamente el crecimiento[36,45].

Para entender mejor la respuesta de la vid ante estos dos factores, que consecuentemente deberían estudiarse conjuntamente, deben definirse ambos conceptos. Por un lado, el concepto de **sequía** hace referencia a un fenómeno meteorológico prolongado que provoca un desequilibrio entre la precipitación y evapotranspiración, siendo capaz de promover el agotamiento del contenido de agua tanto en el suelo como en la atmósfera[28,47]. Así mismo, se considera **estrés térmico** cuando la radiación solar incide en el suelo y eleva la temperatura por encima de la temperatura del aire o cuando la planta es incapaz de disipar el calor inducido en las hojas[47]. Esta radiación provoca un aumento en la movilidad de las moléculas que componen las membranas biológicas, proporcionando fluidez a la bicapa lipídica. Estos cambios, junto con la desnaturalización de proteínas y/o el aumento de ácidos grasos insaturados, aumentan la permeabilidad de la membrana, lo que se demuestra por una mayor pérdida de electrolitos y por lo tanto una pérdida de su termoestabilidad[47].

Como se ha comentado anteriormente, en los últimos años, se ha estudiado el impacto que el cambio climático ha generado en los viñedos, y principalmente se ha observado que afecta directamente a los rendimientos de producción, la calidad de las bayas y la fenología de la vid[49,50]. Lo más llamativo de los estudios es la gran sensibilidad que presentan las plantas cuando el estrés se produce o prolonga en etapas clave o críticas a lo largo de su ciclo vegetativo, sobre todo durante la brotación, floración y maduración, produciendo cambios en los tiempos fenológicos y en la duración del crecimiento[51].

Se ha observado que las vides han desarrollado un acortamiento de las etapas de floración y envero[52]. Algunos autores[53] prevén que en España se produzcan avances de floración de unos 6 días en 2050 y hasta 8 días en 2070, y un avance de envero de unos 13 y 18 días respectivamente. Estos hechos están produciendo un desacoplamiento entre la maduración tecnológica (que se ve más

acelerada) y fenológica (que se ve más retardada)[54] puesto que se produce un envero prematuro, generando mayor acumulación de azúcar y por lo tanto una disminución en la acidez de uva[55]. Todo ello hace que la vinificación produzca vinos con mayor contenido alcohólico[56].

Además, se ha observado que si las excesivas temperaturas se prolongan durante la maduración la planta produce una excesiva acumulación de azúcar y un retraso tanto en la síntesis de compuestos fenólicos como en la madurez aromática de la baya, generando aromas de sobremaduración que a su vez reducen la concentración aromática[57-60] o incluso acumulación de metabolitos secundarios como aminoácidos (AAs)[61-63] o agotamiento prematuro de los ácidos orgánicos[57,58,64].

En cuanto a los cambios fisiológicos, se han observado daños por quemaduras o abscisión en hojas, ramas y tallos, así como una decoloración de las bayas o inhibición de crecimiento de las raíces, llegando a producir envejecimiento de las mismas[47].

Por último, existen otros factores derivados del cambio climático que, aunque no han sido muy estudiados inciden críticamente en el rendimiento de las vendimias. Por ejemplo, las precipitaciones intensas o el granizo pueden causar la pérdida total de las cosechas[34,59,65,66]. Así mismo, si se somete la vid a heladas tardías durante la floración (primavera), se podría ver afectada tanto la calidad como el rendimiento de la cosecha ya que el frío produce la latencia de las yemas[67], disminuye el crecimiento foliar y podría provocar senescencia[68].

1.6 ESTRATEGIAS DE MEJORA EN LOS CULTIVOS DE VID

Debido tanto a la creciente variación climática como a las tasas de crecimiento de la población mundial, la cual se estima que pasará aproximadamente a 10 mMill. en 2050[69], la producción agrícola se está viendo amenazada, y amenaza con no poder satisfacer las necesidades alimentarias[70]. Por otro lado, el uso indiscriminado de fertilizantes químicos, pesticidas y/o herbicidas provoca una pérdida extrema de la diversidad microbiana beneficiosa del suelo[71]. Ambos aspectos deben conjugarse en un imprescindible equilibrio que garantice la sostenibilidad de la vida humana en el planeta.

Con objeto de mitigar estos efectos se han propuesto una serie de recomendaciones metodológicas que permitan adaptar los cultivos. Las estrategias más importantes para mejorar la resistencia y resiliencia de los cultivos incluyen desde un enfoque agronómico y fisiológico hasta una mejora molecular desde el punto de vista genético[72]. También existen técnicas menos agresivas para el medioambiente como podría ser el uso de nuevos tipos de agroquímicos y/o bioestimulantes.

1.6.1 PRÁCTICAS AGRÍCOLAS EFICIENTES

A corto plazo deberían llevarse a cabo soluciones que permitan el crecimiento y desarrollo del viñedo bajo condiciones de restricción hídrica. Para ello, existen una serie de prácticas agrícolas que podrían servir como herramienta para mejorar las producciones agrícolas. Podría manipularse la brotación llevando a cabo un sistema de poda tardía, mediante el cual se retrasa la brotación y se asegura la uniformidad del cultivo, suavizando así el riesgo por heladas primaverales[73].

Así mismo podrían instalarse sistemas de riego, sobre todo en zonas de secano. Existen diferentes sistemas de riego; riego superficial tradicional (vigente en algunas regiones vitivinícolas), riego por aspersión presurizado y el riego por goteo, que es quizás el sistema de suministro de agua más utilizado y eficiente en uso hoy en día en la viticultura[74-76]. Sin embargo, debido a las futuras proyecciones climáticas, se espera una reducción global en la disponibilidad de agua. Este hecho, junto al elevado coste que supone para los agricultores la implantación de sistemas de riego, hace necesario abordar otras estrategias que optimicen la acumulación de agua en el suelo y las raíces.

Entre esas otras estrategias la modificación de los sistemas de labranza mediante el uso de residuos de cultivo, podría favorecer la infiltración de agua, promoviendo a su vez la retención de ésta en el suelo[77].

Otra alternativa podría ser, el uso de cubiertas vegetales entre las hileras del viñedo ya que modera la escorrentía de agua y la erosión del suelo en viñedos inclinados, mejorando la fertilidad del suelo porque permiten el acceso a recursos hídricos adicionales[78]. Sin embargo, este modelo presenta muchas desventajas, siendo el más destacado la competencia que se genera en las raíces en cuanto a la captación de nitrógeno inorgánico y el coste que supone[79].

Una de las técnicas más eficaces es el manejo del dosel que permite mejorar el rendimiento de los cultivos bajo estrés porque mejora la disponibilidad de agua y por lo tanto la calidad de las bayas[80]. El dosel está definido como una técnica agronómica que se utiliza para regular el ambiente alrededor de los racimos, como por ejemplo hojas, brotes y frutos[78]. Así, por ejemplo, Pascual *et al.* (2015) demostraron[81] que controlando el número de brotes se favorece el estado hídrico de la vid, sobre todo, en el momento de vendimia.

Por último, existen estudios que contemplan el uso de prácticas más drásticas que podrían llevarse a cabo para combatir los efectos de la sequía, ya que se centran en estudiar la idoneidad de las regiones vitivinícolas actuales. Algunos de ellos proponen una migración de las regiones vinícolas hacia mayores altitudes[82-84] o incluso se ha propuesto una transformación en la elección de las variedades de uva más idóneas a la climatología de la zona en algunas regiones[85]. Así, por ejemplo, Stock *et al.* (2005)[86] estiman que, para 2050, las regiones mediterráneas europeas como Pisa, Toscana, Alguero o las regiones norteñas como Austria y Alemania requerirán una migración hacia la franja norte. Así mismo predicen que para regiones alemanas como Rheingau o Postdam, se tenderá hacia variedades Cabernet-Sauvignon, Chardonnay o Cabernet Franc respectivamente, supliendo así la variedad por excelencia de la región, Riesling[86]. Además, Jones *et al.* (2005)[87] también examinaron numerosos lugares de Europa, observando cambios en la temperatura y consecuentemente en las características fenológicas en variedades de las regiones españolas como Albariño, Verdejo, Garnacha y Mencía entre otras, proponiendo también un cambio futuro tanto en las variedades de vid a cultivar como en las regiones de cultivo[87].

1.6.2 MEJORA GENÉTICA

Los métodos mencionados anteriormente pueden dar soluciones a corto o medio plazo. Sin embargo, a largo plazo sería más conveniente adaptar el cultivo más drásticamente, incluyendo la sustitución del material vegetal. Para la replantación, es importante una adecuada selección del material vegetal. Para ello, es recomendable hacer uso de técnicas que permiten una adecuada selección como son la ingeniería genética a través de la manipulación genética (MG) o la más empleada hasta la actualidad, la selección clonal[88].

La mejora genética es la ciencia de la aplicación de principios genéticos y de fitomejoramiento, así como la fisiología y biotecnología, para mejorar los cultivos para uso humano[77]. Con estas técnicas se puede producir una transferencia de genotipos que modulen o cambien la expresión y/o la actividad de sus genes para mejorar las aptitudes productivas y la adaptación a las condiciones ambientales[73,89]. Podrían darse dos situaciones; sustitución por distintas variedades de vid o sustitución por distintos clones de la misma variedad, siempre y cuando la selección mejore la tolerancia a las condiciones climáticas[88,90].

A finales del siglo XIX se introdujeron en Europa unos pocos genotipos (especies de *Vitis* o híbridos interespecíficos) capaces de superar la filoxera (*Daktulosphaira vitifoliae Fitch*)[91], pero a día de hoy es necesario estudiar la adaptación de diferentes portainjertos capaces de conferir al vástago una mayor tolerancia al déficit hídrico[92,93], así como transferir rasgos positivos modificando su vigor y fenología[94,95]. Se sabe que el patrón puede alterar la expresión génica en el vástago, especialmente en presencia de estrés, enfermedades o factores limitantes[96,97].

Algunos estudios han investigado la variabilidad genotípica de las respuestas de la vid al estrés hídrico, pero todos ellos se han centrado en el desarrollo de las bayas y su composición en términos de calidad[98-100]. Apenas existe información sobre la respuesta que se produce en las hojas, como la fotosíntesis o conductancia estomática[101].

En la vid, los principales cambios se refieren a varios genes estructurales y factores de transcripción responsables de la biosíntesis de compuestos fenólicos como flavonoides y estilbenos, los cuales se acumulan en las bayas durante la maduración y protegen las hojas y los frutos contra el estrés biótico y abiótico[102]. Por ejemplo, con portainjertos 1103 Paulsen (híbrido obtenido en Sicilia),

considerados tolerantes a la sequía, no se detecta influencia directa sobre su resistencia mediante parámetros fisiológicos, de rendimiento y características de las bayas, pero sí modulan el metabolismo secundario de las bayas[103]. Esta vía fenilpropanoide, responsable de la biosíntesis de flavonoides y estilbenos durante las fases de maduración, tiene múltiples funciones biológicas, sobre todo, protegiendo a las hojas y los frutos y desempeñando un papel contra el estrés biótico y abiótico[103].

Así mismo se han evaluado especies como *Vitis acerifolia rafinesque, Vitis aestivalis michaux, Vitis labrusca linneo, Viti slongii prince, Vitis rotundifolia michaux y Vitis vulpina linneo*, con tolerancia a la cal, bajo pH, salinidad, sequía o temperatura[104,105].

La mayoría de los rasgos relacionados con la tolerancia al estrés son complejos porque están regulados por un elevado número de genes[44,106] y, además, las plantas genéticamente modificadas todavía no son aceptadas en la mayoría de los países europeos, porque se consideran una tecnología con alto riesgo potencial tanto para las personas como para el medio ambiente[77]. Teniendo en cuenta que la única opción es la selección tradicional para la obtención de nuevas variedades, y que conlleva un tedioso trabajo de muchos años, es necesario la búsqueda de otras alternativas que alarguen la vida de las plantas y/o que permitan mitigar el estrés por sequía en los viñedos y promover el crecimiento de la vid y la calidad de la uva, protegiendo los recursos hídricos de las regiones vitivinícolas.

1.6.3 OTRAS ALTERNATIVAS

En los últimos años, se han observado efectos medioambientales alarmantes debido a la contaminación del aire y las aguas subterráneas, así como efectos negativos en la capacidad de retención de agua de los suelos, debido al uso abusivo de fertilizantes químicos o productos fitosanitarios como pesticidas, insecticidas, fungicidas o nematicidas[107-109]. A pesar de las exigentes normativas para su comercialización y uso (Reglamento (CE) nº 1107/2009 del Parlamento Europeo y del Consejo de 21 de Octubre de 2009 relativo a la comercialización de productos fitosanitarios, Diario Oficial de la Unión Europea, 24.11.2009, L 309/1)[110] estos productos pueden llegar a ser nocivos para el medioambiente puesto que producen un agotamiento en la capacidad de retención de

agua en el suelo y su fertilidad, afectando a los seres vivos que habitan en él[111,112]. Además, muchos de estos productos tienen una alta persistencia en el suelo y dañan sus características estructurales.

Debido a las desventajas de los productos agroquímicos, se están estudiando otras alternativas más sostenibles, que protejan la bioseguridad y mejoren los rendimientos de cosecha, como por ejemplo los biofertilizantes, hormonas vegetales y/o bioestimulantes.

1.6.3.1 BIOFERTILIZANTES

Los biofertilizantes se consideran alternativas rentables y ecológicas. Son productos basados en medios biológicos, organismos vivos o sustancias naturales que pueden mejorar la química del suelo y sus propiedades biológicas como la fertilidad o biodiversidad, y estimular el crecimiento de la planta mejorando la disponibilidad de nutrientes[107,109,111].

Por ejemplo, se ha demostrado que la adición de biocarbón[108] (coproducto generado en la conversión termoquímica de biomasa) a los suelos podría ser una buena herramienta para mejorar sus condiciones, reducir el estrés hídrico y aumentar la actividad fotosintética de la planta sin afectar a la hidrofobicidad del suelo. Sin embargo, se asocian varias desventajas que no lo hacen satisfactorio, como son su baja vida útil o su sensibilidad a la temperatura[111].

También se han estudiado algunos fertilizantes de tamaño micrométrico a base de elementos de calcio, magnesio, hierro y silicio, que según un estudio de Kara y Sabir (2010)[113] promueven el crecimiento vegetativo debido a la capacidad que tienen las partículas de penetrar en las hojas a través de los estomas, e impulsar su actividad fotosintética. Así mismo, otro estudio confirma que el uso de fertilizantes a base de calcio de tamaño nanométrico, junto o combinado con extracto de algas *Ascophyllum nodosum* (AN), aplicado a vides de la variedad Narince, mejora el crecimiento de las bayas y el contenido de nutrientes de las hojas[114].

1.6.3.2 HORMONAS VEGETALES

Otra alternativa podría ser el uso de reguladores del crecimiento (PGR) a través de su aplicación exógena a la planta o el suelo. Estas sustancias, comercialmente disponibles, están compuestas por varias clases de hormonas vegetales naturales como auxinas, giberelinas, etileno, citoquininas y ácido abscísico (ABA), así como sus análogos sintéticos, que regulan la biosíntesis y el metabolismo de las plantas promoviendo su crecimiento y desarrollo. Así mismo, modulan la respuesta a las tensiones ambientales[115].

Se sabe que, durante el estrés inducido por sequía, ABA regula el cierre estomático, y su aplicación exógena disminuye el contenido de peróxido de hidrógeno (H_2O_2), es decir, reduce la acumulación de especies reactivas de oxígeno (ROS) tóxicas para la planta[116]. Además, el tratamiento de uvas con ácido abscísico sintético (±-*cis*, *trans*-ABA) después de la floración puede tener efectos positivos, ya que reduce el contenido de taninos de las uvas verdes e impulsa la biosíntesis de taninos y antocianinas durante el envero[117].

Existen estudios sobre la aplicación exógena de otro tipo de fitorreguladores, como los brasinoesteroides, etileno, auxinas, citoquininas y giberelinas para averiguar si tienen implicación directa sobre la maduración de las bayas. Se sabe que algunas de estas fitohormonas actúan como activadores de la división celular y otras como represores del crecimiento[118]. Por lo general se cree que el ABA, los brasinoesteroides y el etileno promueven la maduración a través de interacciones complejas, mientras que las auxinas retrasan algunos procesos asociados a la maduración e interactúa con otras hormonas como ABA y etileno[119].

En cuanto a las fitohormonas que promueven la activación de la maduración de la uva se encuentran por ejemplo los brasinoesteroides. Así, con la aplicación foliar de 2,4-epibrasinolida (Epi-Br) en bayas durante las etapas de desarrollo, se ha descrito un aumento significativo en el contenido de antocianinas en la piel de la baya y una acumulación de azúcar en la pulpa[120,121]. De la misma forma, la aplicación en las bayas, durante el envero, de ácido 2-cloroetilfosfónico (2-CEPA), un compuesto liberador de etileno produce un aumento en la concentración de varios derivados de antocianinas en bayas de Cabernet Sauvignon sin afectar a su peso[122]. Sin embargo, ese efecto

depende en gran medida de la etapa de desarrollo en la que se encuentre la baya en el momento de la aplicación[123].

En cuanto a los retardantes de la maduración, la aplicación antes del envero de auxinas sintéticas como ácido 2-naftalenacético (NAA) y ácido 2-benzotiazol-2-oxiacetico (BTOA), ha demostrado que disminuye la acumulación de antocianinas[124,125].

Por otro lado, se sabe que las citoquininas (CKs) están involucradas en una gran variedad de procesos esenciales para la supervivencia de la planta, sobre todo en la tolerancia al estrés[126] o en la mejora del rendimiento o el tamaño de las bayas. Por ejemplo, se ha demostrado que la aplicación de CKs sintéticas como Forclorfenuron (CPPU) ha mejorado el tamaño de las bayas y el peso del racimo en uva[127]. Sin embargo, su aplicación tiene el inconveniente de ser un compuesto que presenta problemas de persistencia alterando así las características del suelo[128,129]. Otros autores han corroborado que CPPU, a pesar de promover la síntesis de taninos condensados, también produce un retraso en la acumulación de azúcar y antocianinas[130-132]. Así mismo, existen estudios sobre el uso de otras citoquininas sintéticas como Tidiazurón (TDZ) que se han utilizado ampliamente en uva para mejorar el cuajado y/o aumentar el tamaño de la fruta[133].

Por último, aunque no existen muchos estudios sobre su implicación en el control de la maduración de las bayas, las giberelinas han sido utilizadas en uvas sin semilla (partenocarpia) durante las primeras etapas de su desarrollo para estudiar su respuesta al aumento de tamaño y mejorar así el valor económico de los frutos[123,131].

A pesar de los trabajos mencionados anteriormente sobre la regulación hormonal en la maduración de las uvas, éste es un proceso complejo que implica la interacción entre varios reguladores de crecimiento y por lo tanto siguen siendo necesarios otros estudios que muestren evidencias directas entre las vías hormonales durante el desarrollo de la baya.

1.6.3.3 BIOESTIMULANTES

Otra de las alternativas que ha ido ganando consideración en la agricultura sostenible son los bioestimulantes vegetales. Estos productos, son una clasificación diversa de sustancias que, mediante su aplicación directa en el suelo o las hojas, pueden activar ciertos procesos fisiológicos

en la planta que le permiten mejorar la eficiencia del uso de nutrientes, estimulando el desarrollo de las plantas y permitiendo la reducción del consumo de fertilizantes. Además, muchos de ellos pueden contrarrestar el efecto del estrés biótico y abiótico, mejorando la calidad y el rendimiento de los cultivos[134,135].

Debido a la creciente demanda de bioestimulantes, alcanzando el mercado mundial en 2018[136] los 2241 Mill. de dólares, se están introduciendo progresivamente productos innovadores con diferentes bases para satisfacer los requisitos de todo tipo de cultivos herbáceos, hortícolas y vitivinícolas[137].

El principal problema o necesidad que presentan estas sustancias es la regulación del comercio entre los diferentes países, ya que debido a la ausencia de una definición sólida de bioestimulante como grupo discreto de productos, existe una barrera en su desarrollo y en su comercialización[138].

A lo largo de los años, han existido numerosos intentos por concretar una definición, y eso ha supuesto una dificultad a la hora de distinguir claramente los bioestimulantes de otras clases de compuestos, ya que, hoy en día, su composición y efecto es en parte desconocida. Varias propuestas sugieren que los bioestimulantes vegetales pueden definirse por su modo de acción, su origen o en función de su efecto en la productividad de la planta[134].

En 1997, Zhangand Schmidt definió[139] los bioestimulantes como "materiales que, en cantidades diminutas, promueven el crecimiento de las plantas". Más tarde, en 2007, Kauffman *et al.*, definieron[140] que "los bioestimulantes son materiales, distintos de los fertilizantes, que promueven el crecimiento de las plantas cuando se aplican en pequeñas cantidades".

Por último, el consejo Europeo de la Industria de los Bioestimulantes (EBIC), propuso formalmente[141] que "los bioestimulantes vegetales contienen sustancias y/o microorganismos cuya función cuando se aplica a las plantas o a la rizosfera es estimular procesos para mejorar o beneficiar la absorción de nutrientes, eficiencia de los nutrientes, la tolerancia al estrés abiótico, la calidad del cultivo, la vida útil y la conservación después de la cosecha".

En algunos países o regiones administrativas se identifican estos productos como "productos de composición compleja" de sustancias químicas[138]. Desde el punto de vista legal, los bioestimulantes pueden contener trazas de hormonas vegetales naturales, pero no se les debe atribuir su acción biológica porque si no, deberían ser registrados como reguladores del crecimiento vegetal[134].

El Parlamento Europeo considera dos categorías de productos basados en los reglamentos CE nº 2003/2003 y CE nº 1107/2009 respectivamente[110,142]. Por un lado, considera los fertilizantes en función de los nutrientes que proporcionan a la planta y sus formas químicas, incluyendo sus complejos y quelatos[139,143]. Por otro lado, define "Productos Fitosanitarios" como todos aquellos productos vegetales que protejan a la planta de cualquier organismo nocivo o de su efecto, o aquellos productos que influyan en los procesos de vida de la planta, desde su crecimiento sin considerarse un nutriente[139,143].

Más recientemente, el nuevo Reglamento Europeo (UE)[144] 2019/1009 ha definido los Bioestimulantes como "un producto fertilizante de la UE cuya función es estimular los procesos de nutrición vegetal independientemente del nutriente del producto contenido, con el único objetivo de mejorar una o más de las siguientes características de la planta o la rizosfera de la planta: i) eficiencia en el uso de nutrientes, ii) tolerancia al estrés abiótico, iii) rasgos de calidad, o iv) disponibilidad de nutrientes confinados en el suelo o rizosfera"[145]. Esta definición enfatiza como los bioestimulantes se distinguen y especifican en sus funciones agrícolas, y para ello, pueden derivar de una amplia gama de materias primas que tienen diferentes sustancias bioactivas[145].

En España, la legislación vigente, Orden APA/1470/2007, considera estos compuestos como "otros medios de defensa fitosanitaria"[146] y permite su uso abarcando desde agentes de control biológico hasta productos distintos a productos fitosanitarios capaces de potenciar la resistencia de las plantas a los organismos nocivos y protegerlas contra deficiencias no parasitarias[147].

Estas definiciones son muy amplias, ya que los bioestimulantes no son nutrientes en sí pero sí mejoran su absorción[135]. Además, tampoco podrían considerarse únicamente como productos fitosanitarios ya que los bioestimulantes presentan una amplia y compleja naturaleza, producción y uso. Todo ello hace que los bioestimulantes, incluso de la misma naturaleza, difieran en el mecanismo y modo de acción; características únicas de los bioestimulantes que dicha definición no reconoce[143].

Debido a la gran variedad de formulaciones, la clasificación de los bioestimulantes también ha sido un proceso complejo que ha ido evolucionando a lo largo de los años. En 2007, Kauffman *et al.*, clasificaron[140] los bioestimulantes en 3 grupos en función de su fuente y contenido; sustancias húmicas (HS), productos que contienen hormonas (HCP) y productos que contienen aminoácidos

(AACP). Más tarde, Bulgari *et al.*, propusieron[134] una clasificación en función del efecto fisiológico que los bioestimulantes generaban en las plantas, indicando los objetivos fisiológicos y la ruta metabólica implicada.

Pero no fue hasta 2015 cuando Du Jardin[139] proporcionó una amplia clasificación de los bioestimulantes distribuidos en siete grupos diferentes[143].

- *Ácidos Húmicos y Fúlvicos*: son compuestos orgánicos poliméricos que se producen por descomposición de residuos vegetales, animales y microbianos o por la actividad metabólica de microbios del suelo que usan estos sustratos[134,135]. Estos compuestos se extraen de materia orgánica humedecida (turba), de compost y vericompost y de depósitos minerales (leonardita)[134,139,148].

  Existen 3 tipos de ácidos; Húmicos, Fúlvicos y Huminas. Los primeros presentan mayor peso molecular mientras que los segundos, además de no poder ser extraídas del suelo, presentan mayor contenido en oxígeno y menor peso molecular[135,136,147]. Todos estos ácidos presentan una serie de efectos positivos directos e indirectos sobre la planta. Promueven el crecimiento de las plantas porque mejoran la biomasa y la absorción de nutrientes como nitrógeno, hierro, fósforo, potasio o zinc, ejerciendo un efecto similar a la que es atribuida a las fitohormonas auxinas[1343,147,148], mejoran el intercambio de carbono y oxígeno entre el suelo y la atmosfera[137], y estimulando la elongación de los brotes por el aumento de biosíntesis de clorofila, que hace la planta metabólicamente más eficiente[134,139].

- *Elementos inorgánicos*: se han definido los cinco principales elementos beneficiosos inorgánicos, presentes en los suelos y las plantas como sales inorgánicas o en sus formas insolubles[139]: aluminio, cobalto, sodio, selenio y silicio. Estos elementos promueven el crecimiento de las plantas, la tolerancia al estrés abiótico gracias a su acción fungicida y fertilizante y la calidad de sus productos puesto que actúan sobre la eficiencia nutricional[143].

- *Quitosano:* es un derivado acetilado del biopolímero quitina, que se produce tanto de forma natural como industrial[139]. En agricultura, el quitosano se ha usado principalmente como fungicida, pero presenta efectos fisiológicos debido a su capacidad para unirse a componentes

celulares o a receptores específicos involucrados en genes de defensa contra la sequía. Además, afecta a los rasgos de calidad relacionados con el metabolismo primario y secundario[143].

- *Materiales orgánicos complejos*: se obtienen a partir de sustancias muy diversas como abonos, estiércol, extractos de fangos de depuradora, residuos agroindustriales y urbanos y/o tejidos vegetales[143]. Recientemente existen en la literatura estudios en vides de la variedad Airén, que muestran el efecto estimulante sobre polifenoles mediante la aplicación foliar de extractos orgánicos de sarmientos a la hoja. En dichos estudios se observa un aumento del rendimiento del cultivo de vid, así como un impulso en su calidad aromática[149,150].

- *Microorganismos beneficiosos:* existe un gran interés por los microorganismos asociados a las plantas que colonizan las superficies de éstas y los tejidos internos y se conocen como inoculantes microbianos. Estos microbios pueden estar disponibles en la filosfera, rizosfera y endosfera de la planta y, gracias a su interacción con la planta pueden influir su estado de salud y su ecosistema, pudiendo producir efectos beneficiosos o perjudiciales[36,151,152].

  En primer lugar, se definieron las rizobacterias promotoras del crecimiento (PGPR) y las bacterias promotoras del crecimiento (PGPB), ambas aisladas de la rizosfera. Estos compuestos han sido ampliamente estudiados puesto que han demostrado generar resistencia frente a las tensiones bióticas y abióticas[136,153], gracias a la capacidad de asimilar el fósforo para su propio requerimiento a su vez que permiten su disponibilidad en el suelo[154,155]. Además, promueven la síntesis de ciertas fitohormonas endógenas de las plantas, y favorecen su crecimiento y desarrollo a través de respuestas metabólicas que reducen los efectos negativos de la sequía, o el ataque de patógenos[136,156,157]. Las PGPRs más estudiadas son distintas especies de los géneros *Burkholderia, Bacillus, Pseudomonas, Serratia* y *Strptomyces*, *Azospirillum*y *Bradyrhyzobium*[36,151,152]. Por ejemplo, en vid se sabe que *Burkholderia phytofirmans* puede generar un aumento en el crecimiento de raíces secundarias, así como mejorar la resistencia a bajas temperaturas, *Pseudomonas sp.*, *Pantoea sp.* y *Acinetobacter sp.*, mejoran la resistencia contra *Botrytis cinerea*, y *B. licheniformis* y *P. fluorescens* generan tolerancia a la sequía en vid[152].

También hay que considerar los hongos beneficiosos de la rizosfera que promueven el crecimiento de las plantas (PGPF)[158]. Estos hongos se pueden aislar del suelo, de las plantas, de residuos vegetales, de agua o de abonos compostados[136]. Los géneros más estudiados son *Candida*, *Gliocladium y Thrichoderma*[151] el cual tiene mayor capacidad para promover el crecimiento y las defensas de las plantas[36]. Así mismo las micorrizas formadoras arbusculares (AFM) protegen contra el estrés biótico y abiótico[36].

Por último, las levaduras beneficiosas que se encuentran en la filosfera y rizosfera son capaces de colonizar las hojas y controlar muchos patógenos mediante activación de defensa sistémica[159]. También pueden promover el crecimiento de las raíces y la solubilización del fosfato[160].

- *Hidrolizados de Proteínas (PH):* son una mezcla de péptidos, oligosacáridos y AAs de bajo peso molecular que se obtienen por hidrólisis química y enzimática de proteínas procedentes de subproductos agroindustriales de origen vegetal y/o animal[136,161]. Mientras que los de origen vegetal están compuestos por carbohidratos solubles y fenoles, los de origen animal carecen de carbohidratos, fenoles y fitohormonas[162]. También se incluyen betaínas, poliaminas y AAs proteicos y no proteicos[136,161].

  Los PHs son capaces de mejorar el funcionamiento fisiológico de las plantas, aumentando el crecimiento de los tejidos, la absorción de nutrientes y la tolerancia al estrés biótico y abiótico. En la vid, se ha demostrado que los PHs son capaces de mejorar el contenido de antocianinas, compuestos fenólicos y antioxidantes de las hojas y reducir la propagación de Mildiu de la vid (*Plasmopara vitícola*) y la putrefacción del racimo[161,163-165], ya que mediante su uso se aumenta la asimilación de nitrógeno a través de la estimulación del metabolismo del carbono y nitrógeno[136].

  Las poliaminas (PAs) son policationes ubicuos de bajo peso molecular que juegan un papel esencial en los procesos regulatorios y celulares como son el crecimiento y desarrollo de las plantas, senescencia, respuesta al estrés[164] y abscisión de racimos de uva[167,168]. Las PAs son también moléculas de señalización que interactúan con otros metabolitos, incluidos los

compuestos protectores del estrés y las hormonas de las plantas, y confieren beneficios agrícolas como la mejora del contenido de fitonutrientes y la calidad de la uva[169].

Las PAs más estudiadas y que más comúnmente se acumulan en las plantas que sufren estrés son putrescina (Put), espermidina (Spd), espermina (Spm) y en ciertas ocasiones, en algunas especies concretas de plantas, también termoespermina (tSpm)[166]. Aunque su metabolismo y por ente su acumulación se dé más como una respuesta de la planta al estrés, no está claro cómo su síntesis, catabolismo o la combinación de ambos está involucrada en los procesos de tolerancia al estrés de la planta[167,170]. En algunas especies otro tipo de factores como la fase del desarrollo de la planta en la que se produzca el estrés o la propia intensidad del estrés podrían influir en el contenido endógeno de PAs[170]. Por lo que puede ocurrir que en ciertas plantas no se observen fluctuaciones del contenido de PAs o incluso se reduzca su contenido endógeno de alguna de ellas[169].

Por ejemplo, se ha confirmado que, en uvas, se reduce la síntesis de PAs dando lugar a la acumulación de *L*-arginina (Arg). Sin embargo, el catabolismo de las poliaminas es activo durante la maduración de la uva y produce acumulación de ácido *γ*-aminobutírico (GABA) debido a la actividad diamina oxidasa[171]. Dicho catabolismo puede constituir una fuente de ROS que a su vez activa la respuesta oxidante de la planta como defensa contra el estrés biótico y abiótico[171].

Otra vía importante que utilizan las plantas como respuesta al estrés es la acumulación de AAs no proteicos como ácido *β*-aminobutírico (BABA) y GABA, o proteicos como prolina (Pro) y arginina (Arg), relacionados con el metabolismo de las PAs y que actúan como moléculas de señal endógenas también relacionadas con la respuesta oxidativa de las plantas[136].

- *Extractos de algas marinas (SWE):* son un grupo de compuestos naturales purificados que incluyen polisacáridos complejos como laminarina, alginatos, carragenanos, betaínas y hormonas vegetales[139]. Las macroalgas marinas comprenden casi 10000 especies, pero los extractos comerciales actuales se fabrican principalmente de algas pardas (*Phaeophyta*), cuyos principales géneros son AN, *Laminaria spp, Ecklonia máxima y Fucus serratus*[172], aunque

también se emplean macroalgas rojas (*Rhodophyta*) y verdes (*Chlorophyta*) que representan el 10% de la producción marina[135,172,173].

Hasta el momento las investigaciones estaban centradas en su actividad pesticida cuando eran añadidos al suelo debido a sus propiedades como acondicionadores[173]. Sin embargo, en los últimos años se está centrando la atención en sus propiedades bioestimulantes, las cuales se creen son debidas a la presencia de hormonas vegetales o reguladores del crecimiento como citoquininas o auxinas que pueden activar genes endógenos sensibles al estrés[139]. Así mismo, se cree que, gracias a la alta concentración de AAs, vitaminas y otros metabolitos secundarios bioactivos considerados solutos compatibles, el uso de este tipo de bioestimulantes pueden proteger a la planta bajo estrés osmótico, mejorando su balance hídrico, y protegiendo los tejidos vegetales contra ROS inducidas por estrés[46,177].

Existen varios estudios que informan del efecto bioestimulante que los extractos de AN producen en distintas variedades de vid cuando se aplican, incluso en concentraciones bajas, en forma de pulverización foliar, ya que pueden estimular numerosos rasgos ecofisiológicos y bioquímicos, mejorando el metabolismo de la piel de las bayas, la floración y su rendimiento[46,176]. Además, se reporta que dichos extractos mejoran la fotosíntesis porque estimulan la producción de clorofila[155,174], la síntesis de ácidos nucléicos, la absorción de iones y el crecimiento vegetativo[114,177] y desarrollo de las raíces[175]. De esta forma, mejoran la disponibilidad de nutrientes, e incrementan la tolerancia a diversos factores abióticos como la sequía, salinidad y congelación[178] y conceden mayor resistencia a hongos, bacterias y virus como por ejemplo Botrytis (*Botrytis cinérea)* o Mildiu de la vid[155,173,174,179] y afectan a la biosíntesis de hormonas vegetales[178].

Debido a las diversas composiciones de los bioestimulantes, es difícil definir un mecanismo de acción común a todos ellos, e incluso para algunos de ellos aún no se conoce. Es importante definir el modo de acción como un efecto específico sobre un proceso bioquímico o regulador de una molécula bioactiva concreta, mientras que el mecanismo de acción describe todos los eventos bioquímicos posteriores a la aplicación[138].

El efecto estimulante ejercido sobre las plantas puede depender de acciones sinérgicas de más sustancias, y no sólo de los componentes individuales[138]. Sin embargo, se conocen algunos modos de acción (*Fig. 1.4*) que se centran en los impactos positivos en la productividad de las plantas mediante la mejora de la fotosíntesis, el retraso de la senescencia o la modulación de fitohormonas, la absorción de nutrientes y agua y la activación de genes responsables de la resistencia al estrés biótico y abiótico y la fenología de la planta[135,138].

Uno de los objetivos de este trabajo es la aplicación de extractos de algas marinas de diversas marcas comerciales, cuyo mecanismo de acción está perfectamente definido. La tolerancia al estrés está modulada por un potencial hídrico foliar negativo y un mantenimiento de la conductancia estomática debido al aumento en los flujos de iones K^+ y Ca^{2+} en los estomas generando dicha tolerancia[135,177].

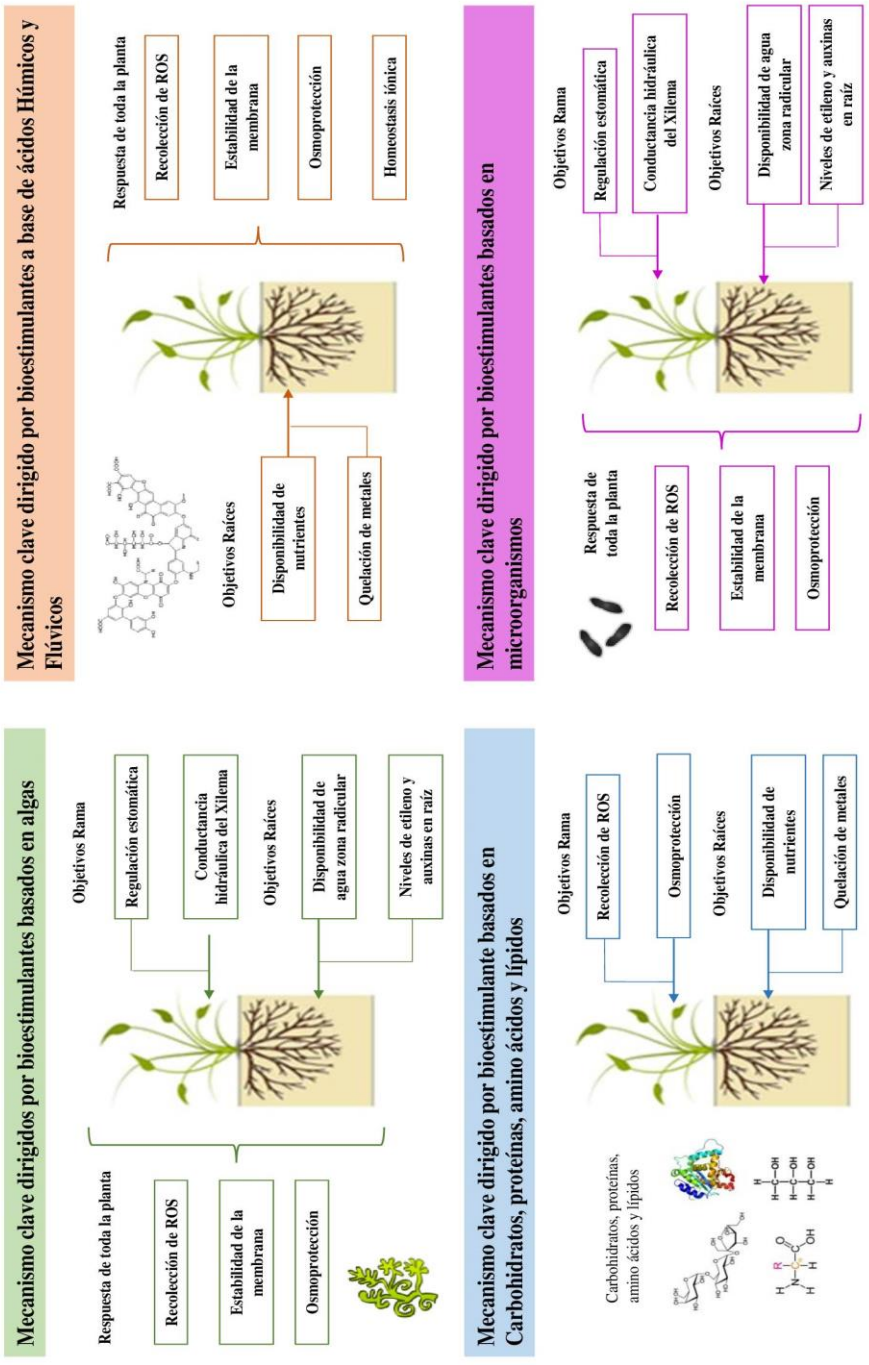


Figura 1.4. *Principales mecanismos clave en función de la base de bioestimulante aplicada a la planta. Adaptado de " Van Oosten, M. J.; Pepe, O.; De Pascale, S.; Silletti, S.; Maggio, A. The Role of Biostimulants and Bioeffectors as Alleviators of Abiotic Stress in Crop Plants. Chem. Biol. Technol. Agric.* **2017**, *4 (1), 1–12* " [135].

1.7 ANALITOS DE SEÑALIZACION EN RESPUESTA AL ESTRÉS HÍDRICO

Muchos de los metabolitos primarios como AAs, AcOrg y carbohidratos, son considerados solutos compatibles que la planta sintetiza bajo condiciones de estrés, ya que funcionan como osmolitos que ralentizan la pérdida de agua y que pueden actuar como moléculas señal que activan la respuesta de la planta a estreses como son el aumento de la temperatura y/o el estrés hídrico[180].

1.7.1 ÁCIDOS ORGÁNICOS

Los ácidos orgánicos (AcOrg) están involucrados en diversas rutas bioquímicas como glucólisis, fotorrespiración, ciclo del glioxilato, fotosíntesis en plantas C4 y ciclo de Krebs. Generalmente la planta los utiliza como fuente de energía, como intermediarios en el metabolismo de carbono dentro de las células vegetales y/o como moduladores del transporte a través de membranas o mensajeros de señalizaciones[181].

Recientemente se ha indicado que los ácidos orgánicos intervienen en la conductancia estomática (*i.e.* ácido málico), la biosíntesis de AAs (ciclo de Krebs), la interacción planta-microbio, el equilibrio de exceso de cationes o incluso los mecanismos para hacer frente al déficit de nutrientes[182,183]. Así mismo están involucrados en la respuesta de la planta al estrés abiótico debido a su metabolismo en el citosol y su capacidad metabólica participando como solutos activos para el ajuste osmolítico[184].

Además, su biosíntesis, acumulación, transporte y exudación radicular aumenta drásticamente en respuesta a las condiciones ambientales de estrés[183].

Como ya se ha comentado anteriormente, las condiciones ambientales están fuertemente relacionadas con una fenología de desarrollo más temprana debido a las condiciones ambientales cambiantes, que están afectando directamente a la calidad de la uva con una notable disminución en la concentración de ácidos orgánicos[184,185]. Sobre todo, el ácido málico y el ácido tartárico, que predominan en todas las etapas del desarrollo, tienen una influencia significativa en la acidez y pH del vino[184].

Es conocido que ácido málico (Mal), cítrico (Cit) y tartárico (Tart) son los ácidos predominantes en bayas, sin embargo, el ácido tartárico no es metabolizado a través de la respiración como el ácido málico y por ello permanece constante durante la maduración[186]. Durante la fermentación aparecen otros ácidos orgánicos importantes como; ácido succínico (Succ), derivado intermedio del metabolismo de las levaduras producido durante el ciclo de Krebs, o el ácido acético (Acet), producido durante la fermentación[165]. Sin embargo, la producción de todos estos metabolitos está muy influenciada por el impacto del estrés osmótico.

1.7.2 AMINOÁCIDOS

Los AAs tienen funciones importantes en la tolerancia al estrés, ya que estimulan el crecimiento celular y su aplicación exógena tiene efectos positivos sobre el crecimiento y el rendimiento de las plantas, puesto que actúan como osmolitos que regulan el transporte de iones, la desintoxicación de ROS, la apertura de estomas y protegen la integridad de la membrana estabilizando enzimas y/o proteínas[180,187].

El primer aminoácido del que se tiene información por su acumulación bajo condiciones de estrés es Pro[188]. Está considerado un marcador de tolerancia al estrés que, generalmente se acumula en el citoplasma y está involucrado en la regulación osmótica de la planta, y actúa como una molécula de señalización para modular el funcionamiento de la mitocondria. Además, debido a su actividad antioxidante está involucrado en la estabilización de las membranas mediante la acción amortiguadora sobre el potencial redox de la célula en condiciones de estrés[180,189].

Se ha demostrado que Pro se acumula en los tejidos de hoja como respuesta a la retención de agua de las plantas, pero no en todas las especies. Algunas especies producen el ajuste osmolítico mediante acumulación de asparagina (Asn), glutamina (Gln), alanina (Ala), ácido aspártico (Asp) y ácido glutámico (Glu), por lo que la acumulación de Pro no sólo debería utilizarse como indicador de la respuesta a la tolerancia de déficit hídrico. Por otro lado, se ha comprobado que la Arginina (Arg), precursor de las PAs, es un metabolito importante durante las etapas de crecimiento y maduración del fruto y/o la senescencia de la hoja, y está implicado en la respuesta al estrés abiótico y la regulación en la asimilación de nitrógeno[192,193].

Otro AA que controla la respuesta al estrés es GABA, un aminoácido no proteico de cuatro carbonos que funciona como molécula señal[194]. Sus funciones principales son coordinar el equilibrio Carbono/Nitrógeno o regular el pH citosólico[195]. GABA es considerado un metabolito oxidativo y, como soluto compatible, actúa como regulador osmótico, mejora la acumulación de Pro[196] y, es capaz de mediar una respuesta de inanición en las células vegetales y en defensa contra insectos[197,198]. Además, su síntesis vía glutamato libera CO_2 que la planta puede usar en condiciones de estrés, cuando la fotosíntesis se ve reducida. Este AA, ya se ha mencionado anteriormente, puede ser obtenido mediante la degradación de PAs[194].

Cabe mencionar que desde hace más de 50 años se sabe que la aplicación exógena mediante pulverizaciones foliares de BABA (isómero de GABA) aporta beneficios en la respuesta de las plantas al estrés generado por las altas temperaturas y falta de agua mediante su influencia en la ruta metabólica del ABA[199-201]. Generalmente BABA no induce directamente la respuesta de defensa, pero prepara a la planta para que reaccione más rápido o más fuerte (imprime) frente a un estrés dado[202]. Además, BABA es considerado un metabolito que mejora la resistencia de la planta para combatir las enfermedades producidas por hongos como Mildiu de la vid o *Botrytis cinérea* a través de la activación de genes involucrados en la resistencia sistémica adquirida de la planta (SAR) cuya expresión depende de los niveles de ácido salicílico (SA)[203-205].

1.7.3 CARBOHIDRATOS

Los carbohidratos son fotoasimilados, cuya implicación principal en las plantas es servir como fuente de energía y como nutriente para los tejidos vegetales[206]. Los azúcares se caracterizan por ser sustancias solubles de bajo peso molecular, que se pueden almacenar de forma temporal en hojas y pasar después a ser usados en condiciones de estrés o de forma permanente como en troncos y raíces[207]. Tanto el almidón como los azúcares solubles son indispensables para el crecimiento y el almacenamiento como fuente de energía[180].

Los azúcares solubles, principalmente fructosa (Fruc), glucosa (Gluc) y sacarosa (Sac), se utilizan como recursos para la respiración y el metabolismo, además de ser considerados moléculas de señalización para controlar la expresión génica de procesos biológicos como la fotosíntesis y el crecimiento de las plantas[208-210]. En este sentido, está descrito que las altas temperaturas regulan

negativamente la acumulación de carbohidratos solubles como glucosa y sacarosa en las plantas debido a los cambios producidos en la ruta metabólica de la sacarosa[180]. A su vez, la sacarosa puede desempeñar un papel como osmoprotector para mantener la integridad de la membrana celular y su función bajo estrés por calor o sequía, así como eliminar ROS[180,211].

En la vid, los carbohidratos solubles como Fruc, Gluc y Sac se encuentran en el tronco y las raíces, influyendo en su longevidad, potencial de calidad, desarrollo del área foliar, el crecimiento de los brotes e inducción de la floración[212]. Además, están involucrados en la asimilación de carbono cuando la planta está sometida a restricción de agua[213]. Cuando se produce estrés por sequía, se produce la acumulación de carbohidratos en hojas, debido a la inhibición de su transporte hacia las raíces, pudiendo provocar con el tiempo la mortalidad de la planta[214]. Por lo tanto, el seguimiento de los cambios en estos compuestos entre parte aérea y raíz podrían ser un indicador de respuesta al estrés hídrico severo[215].

1.7.4 Interconexiones del Metabolismo Primario en Condiciones de Estrés

Algunos estudios metabólicos[216,217] han reportado la interconexión entre el contenido y la acumulación de azúcares, AcOrg y AAs en diferentes partes de la vid bajo condiciones de estrés hídrico.

Por un lado, los azúcares solubles, principalmente Fruc, Gluc y Sac juegan un papel importante en el desarrollo de la composición final de la baya. Además, a través del ciclo glucolítico (respiración) y el ciclo del ácido tricarboxilico (TCA) (*Fig. 1.5*), estos metabolitos pueden aportar energía (ATP), potencia reductora (NADPH), y son precursores para la síntesis de AcOrg, AAs y metabolitos secundarios de defensa y aromáticos que regulan la acidez de la fruta[218,219].

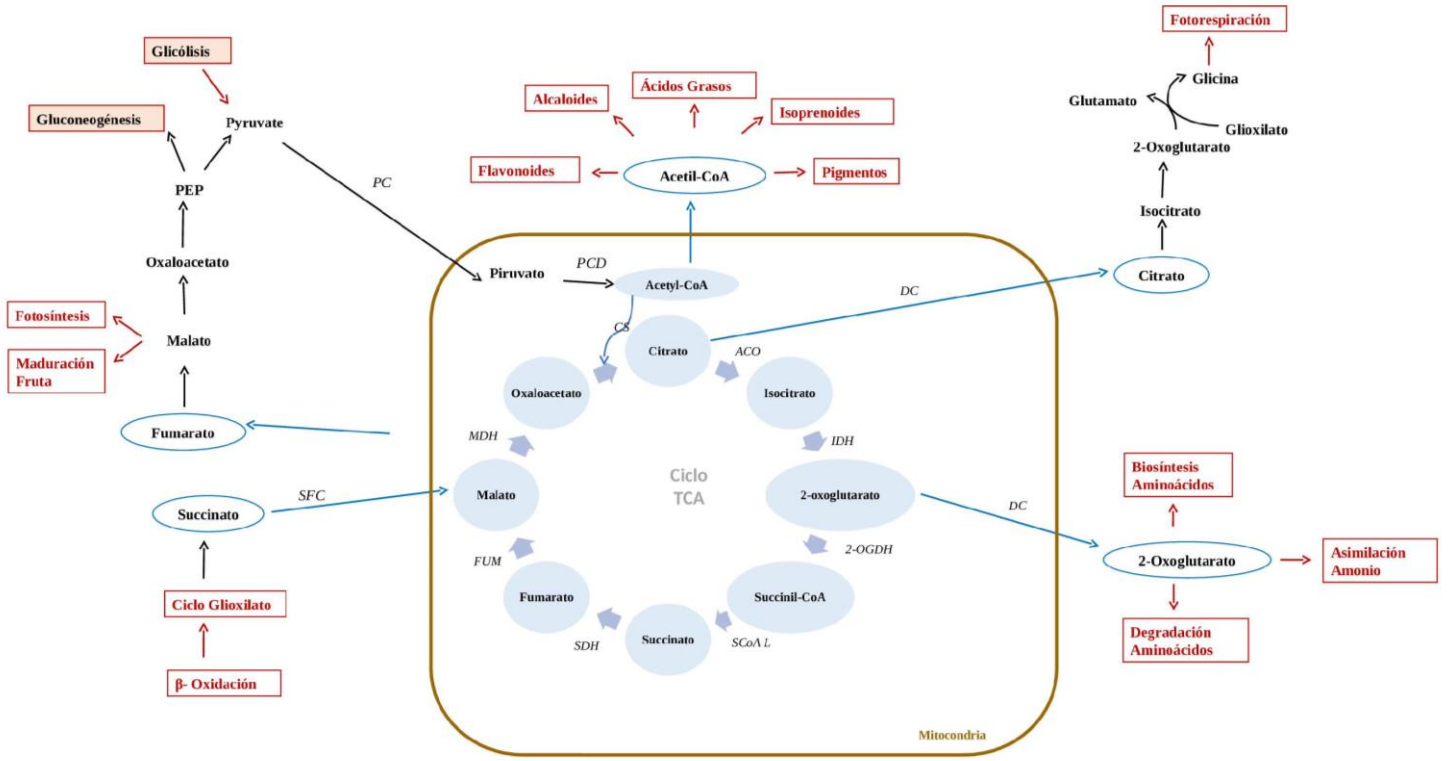


Figura 1.5. *Resumen esquemático del ciclo del TCA y su vías convergentes y divergentes. Adaptado de " Araújo, W. L.; Nunes-Nesi, A.; Nikoloski, Z.; Sweetlove, L. J.; Fernie, A. R. Metabolic Control and Regulation of the Tricarboxylic Acid Cycle in Photosynthetic and Heterotrophic Plant Tissues. Plant, Cell Environ.* ***2012****, 35 (1), 1–21" [219].*

No sólo la acumulación de azucares está fuertemente influenciada por las temperaturas durante las diferentes etapas del ciclo vegetativo, sino también el contenido de AcOrg. Entre los primeros análisis completos de la composición de AcOrg en uva, se han llegado a identificar hasta 23 ácidos orgánicos en el tejido de la vid, donde destacan el Tart, Mal, Cit, ascórbico, oxálico (Oxal) (en forma de cristales de calcio), así como Succ, Fum, y otros ácidos orgánicos en pequeñas cantidades[220]. Sin embargo, los ácidos orgánicos predominantes son Mal y Tart, los cuales pueden llegar a representar el 90% de la acidez total de la baya y contribuyen en el vino final producido[221].

El primer estudio del que se tiene constancia en hojas de vid sobre las rutas metabólicas de Mal y Tart data de 1958[222], donde se fueron expuestas hojas de parra cortadas marcadas con ^{14}C a periodos variables de luz y oscuridad, dilucidando así que las vías sintéticas de ambos metabolitos no estaban ligadas entre sí, y por lo tanto proceden de diferentes partes dentro de la baya. La acumulación neta de Tart se produce en la primera etapa del desarrollo de la baya, producido en el citosol y en los apoplastos debido a la degradación de ácido ascórbico[221,223] aunque generalmente no es utilizado en las vías metabólicas primarias[224]. Se cree que prácticamente permanece intacto durante la maduración debido a la formación de una sal estable de bitartrato de potasio[223].

En contraste, Mal se forma antes del envero en uvas verdes a partir del metabolismo de los azúcares y a través de la fotosíntesis[225,226], siendo metabolizado activamente durante la etapa de maduración de la vid[225]. Justo después del envero y antes de la etapa de maduración de la uva, Mal se vuelve disponible para el catabolismo, a través de vías como el ciclo del TCA (a través del malato aportado), la respiración, la gluconeogénesis, fermentación y por la producción de compuestos secundarios[221,224,227,228], donde queda como fuente de carbono para la acumulación de azúcares e inhibición de la glucólisis.

Existen ciertos factores como son la temperatura, la luz, la especie, la disponibilidad de agua o la carga del fruto, que condicionan el contenido de AcOrg disponible para el ciclo de Krebs[229]. Sin embargo, los estudios aún necesitan determinar inequívocamente la bioquímica y los mecanismos moleculares por los cuales se produce una mayor degradación del malato en respuesta a la temperatura[185].

Por otro lado, la importancia que los AAs tienen en el viñedo es bien conocida, ya que juegan un papel esencial en el metabolismo vegetal, tanto como productos primarios en la asimilación de

nitrógeno inorgánico como en el metabolismo secundario como precursores de proteínas y ácidos nucléicos[191], o nutrientes para el desarrollo de levaduras que realizan la fermentación alcohólica y como precursores de compuestos aromáticos [230-233].

Los AAs libres representan la parte más importante del nitrógeno total en mostos y vinos, aproximadamente entre el 30 y 40%[230,234], aunque su contenido en uvas depende de factores como la variedad de cultivo, la fertilización del suelo, el tipo de suelo y su humedad, el manejo del viñedo, las condiciones ambientales, época de crecimiento, estado sanitario, madurez o portainjerto[234-241]. Generalmente, la asimilación de nitrógeno en la vid se produce en las hojas, donde se transforma en nitrógeno orgánico a través de la Gln la cual se transforma a su vez en Glu a través de la acción de la enzima Gln sintetasa (*Fig. 1.6*). El Glu es el principal compuesto implicado tanto en la asimilación como en la disimilación de amoníaco y la base para la síntesis de GABA, Arg y Pro, así como precursor de la síntesis de clorofila en las hojas en desarrollo[6,244,243].

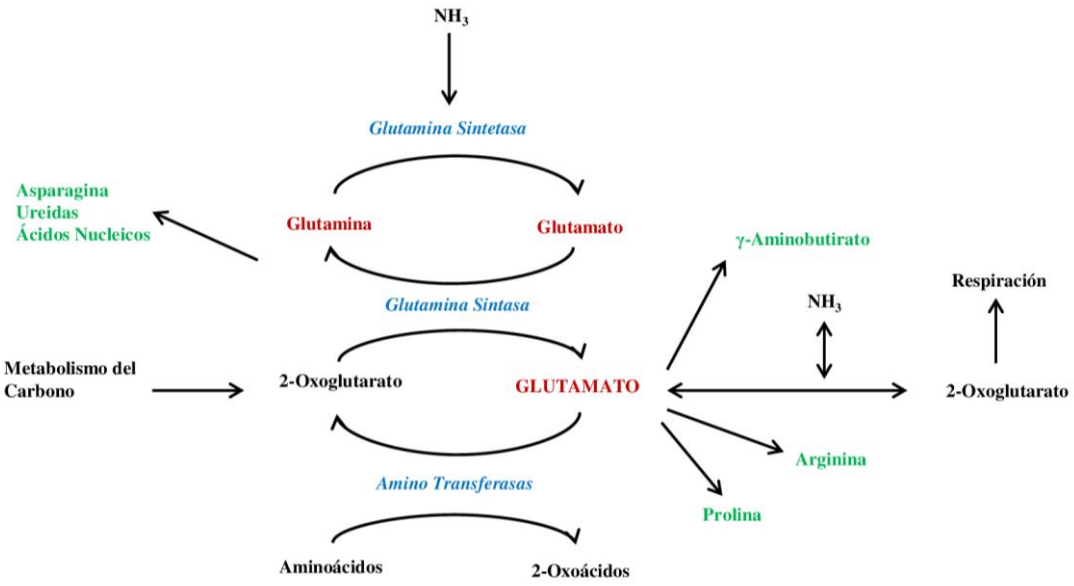


Figura 1.6. *Síntesis y metabolismo de glutamina en plantas. Adaptado de "Forde, B. G.; Lea, P. J. Glutamate in Plants: Metabolism, Regulation, and Signalling. J. Exp. Bot.* ***2007****, 58 (9), 2339–2358"*[242].

Finalmente, algunos AAs se degradan en la mitocondria o el citosol, hacia precursores o intermedios del ciclo del TCA[197]. La *Fig. 1.7* muestra la degradación de AAs hacia el ciclo del TCA y la *Fig. 1.8* muestra una ruta de degradación extendida de los intermedios del ciclo del TCA (resaltados en azul) junto con las rutas de síntesis de AAs a partir de otros AAs precursores (líneas discontinuas).

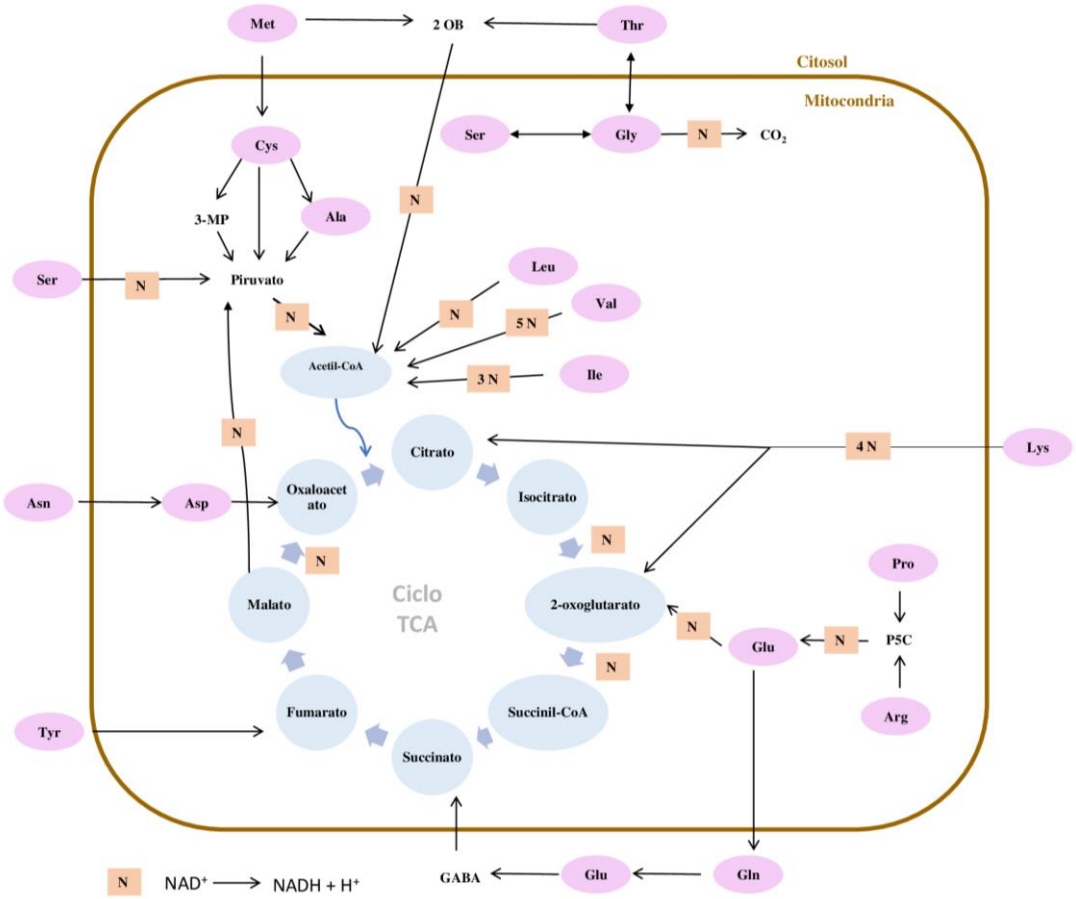


Figura 1.7. *Degradación de aminoácidos (AAs) hacia intermedios del ciclo del TCA junto con la ganancia de nitrógeno. Adaptado de "Hildebrandt, T. M.; Nunes Nesi, A.; Araújo, W. L.; Braun, H. P. Amino Acid Catabolism in Plants. Mol. Plant.* ***2015****, 8 (11), 1563–1579"*[196].

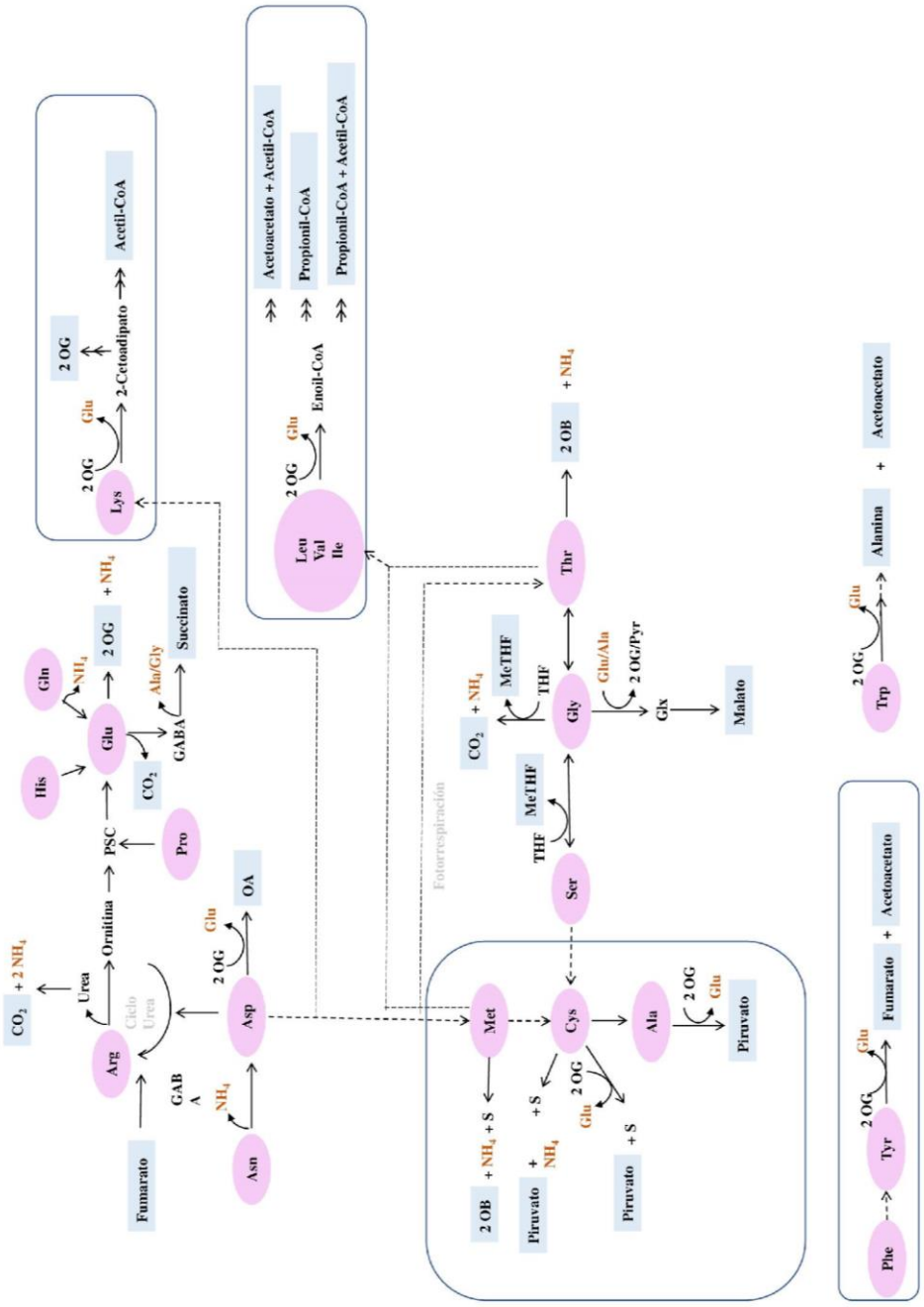


Figura 1.8. *Reacciones de degradación de AAs completas hacia precursores del ciclo del TCA. Adaptado de " Hildebrandt, T. M.; Nunes Nesi, A.; Araújo, W. L.; Braun, H. P. Amino Acid Catabolism in Plants. Mol. Plant.* ***2015****, 8 (11), 1563–1579" [196].*

1.8 ANALITOS DE SEÑALIZACION EN RESPUESTA AL ESTRÉS HÍDRICO

Dada la importancia que estos metabolitos primarios tienen en viticultura y en los productos vitivinícolas finales, su determinación y cuantificación a través de diversas metodologías analíticas ha sido de gran interés. Gracias a la evolución del análisis instrumental su determinación presenta una notable consideración como herramienta para determinar el estado hídrico de *Vitis vinifera* L.

1.8.1 ÁCIDOS ORGÁNICOS

Existen diversas metodologías analíticas, que en menor o mayor medida se han empleado para la cuantificación de esta familia química. La cromatografía de líquidos (LC) es la técnica más utilizada. La separación cromatográfica más habitual se realiza, con columnas de fase reversa (RP), que, generalmente suelen ir acopladas a detectores de matriz de diodos (DAD). Está técnica se ha utilizado en derivados de uva[244-248] y hoja[249-251] y, algunos autores además la han empleado para medir la trazabilidad geográfica del vino[252]. También se ha acoplado a detectores de dispersión de luz evaporativa (ELSD)[253] y de espectrometría de masas en tándem (MS/MS)[254] o tiempo de vuelo (TOF-MS) para su determinación en hojas[255].

Por otro lado, las columnas de intercambio iónico basadas en derivados amino o hidrógeno acopladas a un detector DAD también son habituales para la determinación de ácidos orgánicos[256-258], y se sabe que son una alternativa para mejorar la resolución de ácidos ácido acético y cítrico[259]. Este tipo de columnas se han empleado en la determinación simultánea de estos metabolitos primarios para estudiar el perfil metabólico en diferentes variedades de vino[260-262].

Una variante descrita en literatura es la cromatografía iónica con conductividad suprimida[263,264] para muestras que contienen baja concentración de ácidos orgánicos. La cromatografía de capa fina (TLC) también ha sido descrita por Kliewer[220] para el estudio del perfil de azúcares y ácidos orgánicos en diferentes tejidos vegetales de *Vitis vinifera* como hojas, raíces, corteza, frutos y madera.

Esta familia de metabolitos primarios presenta baja aplicación en el uso de la cromatografía de gases (GC) para su determinación[259], ya que requieren de una etapa de derivatización para mejorar su volatilidad a través de derivados de alquil-éteres, alquil-ésteres o la trimetilsililados[259,265-269], así como de un aislamiento previo mediante el uso de resinas de intercambio iónico o cartuchos de extracción en fase sólida[259,265].

La resonancia magnética nuclear (RMN) se ha empleado en menor medida para la caracterización de ácidos orgánicos. No obstante, cabe destacar el trabajo realizado por Díaz De Cerio et *al.* (2018)[255] en hojas de *Annona Chermila L.* combinádola con cromatografía líquida.

La técnica de electroforesis capilar ha sido empleada en muestras vegetales de vid[270-272] o zumos de uva y vino[259]. Mayormente se ha utilizado para la medida, en vinos, del aumento de coenzima NADH y NADPH que absorben en regiones cercanas a 340 nm[259]. Además, una ventaja que presenta esta técnica es la posibilidad de la determinación de isómeros D y L, aunque las mediciones simultáneas de ácidos orgánicos no son posibles[245] a menos que se utilice el análisis de inyección de flujo (FIA)[273-275].

Por último, también se han utilizado metodologías espectrofotométricas basadas en la reacción de ácidos orgánicos con compuestos que mejoran la formación de un complejo coloreado, como el vanadato de amonio, medibles en la región de 420 y 540 nm. Sin embargo, estos métodos son laboriosos y no existen referencias en literatura para la determinación de ácido succínico, cítrico o acético[259].

1.8.2 AMINOÁCIDOS

Existen dos técnicas que se han empleado por excelencia para la determinación y cuantificación de este grupo de analitos: la cromatografía gaseosa y la cromatografía líquida de alto rendimiento (HPLC).

Aunque los AAs pueden detectarse directamente por UV-Visible a longitudes de onda comprendidas entre 190 nm y 210 nm, la mayoría de los disolventes absorben en dicha región, por lo que es necesaria su derivatización. La derivatización puede tener lugar antes de la separación cromatográfica (pre-columna), después de la separación cromatográfica (post-columna) o en la propia columna[276]. Algunos de los derivatizantes más comunes utilizados en GC y cromatografía

líquida (LC) que se han descrito son nihidrina, cloruro de dansilo (Dns-Cl), cloruro de dabsilo (DABS-Cl), 1-fluoro-2,4-dinitrobenceno (FDNB), fenilisotiocianato (PTIC), ortoftaldehido (OPA), cloroformiato de 9-fluorofen-9-metilo (FMOC), propanodiato de dietilo y carbamato de 6-aminoquinolil-N-hidroxisucinimidilo (AQC)[276]. La nihidrina se utiliza generalmente para la determinación cuantitativa de AAs después de que se hayan separado en su forma nativa mediante cromatografía de intercambio iónico. Por lo tanto, esta derivatización es exclusivamente del tipo post-columna. El cloruro de dansilo se ha empleado para AAs libres, hidrolizados o la determinación de péptidos con derivatización previa a la columna, y se detecta por fluorescencia (λex. 360 nm, λem. 470 nm). Los derivados de dabsil presentan un máximo de absorbancia a 420 nm y se separan mediante métodos pre-columna. FDNB se ha utilizado para la caracterización de AAs terminales o péptidos con derivatización previa a la columna formando compuestos fácilmente detectables por fluorescencia. El fenilisotiocianato se emplea con AAs secundarios como prolina e hidroxiprolina y se limita la detección a la región ultravioleta (254-269 nm). AQC es detectable por fluorescencia y es ampliamente utilizado en LC de fase inversa. Sin embargo, los derivatizantes más utilizados son OPA y FMOC. Por un lado, OPA se utiliza tanto antes como después de la columna y desarrolla fluorescencia cuando reacciona con AAs primarios. Por otro lado, FMOC se utiliza en la determinación de AAs primarios y secundarios precolumna y sus derivados pueden detectarse por monitorización UV a 330 nm o fluorescencia (λem. 430 nm)[276,277].

La mejora del análisis instrumental ha permitido; i) obtener derivatizaciones rápidas antes de la columna, ii) detección y cuantificación simultánea de AAs libres primarios y secundarios en soluciones acuosas con, iii) mayor sensibilidad y precisión, y iv) en un menor tiempo de análisis[276,278].

Generalmente, en cromatografía de gases, los dos detectores más utilizados son el detector de ionización de llama (FID) y el detector de espectrometría de masas (MS). Con este último detector se pueden citar numerosos estudios entre los que caben destacar la determinación de la evolución del vino a base de Cabernet Sauvignon durante su crianza[266], la autenticación de vinos a través del perfil de AAs[279,280], la determinación simultánea de AAs, aminas biogénicas y poliaminas en vino[268,278,281], en mosto y vino de Oporto[282,283] o el cribado de perfiles de AAs en diferentes vinos usando una columna quiral[284].

Dentro de la cromatografía liquida de alta resolución, se han utilizado columnas de fase reversa, fase normal (NP), interacción hidrofílica (HILIC), intercambio iónico (IEC) o de exclusión molecular (SEC)[276,285]. Los detectores más comúnmente empleados con LC son los detectores Uv-Visible[286,287], FLD[288,289] o MS[290]. Generalmente, el perfil de AAs a través de la cromatografía de intercambio iónico se ha utilizado para el estudio del origen, variedad o portainjerto[291] o en el estudio de isómeros D y L de AAs en plantas y vinos[292,293]. Aunque no es muy habitual, el uso de la cromatografía líquida a través de la magnetocromatografía líquida de alto rendimiento (HPLMC) en esta área, también ha sido utilizada para la determinación de AAs en vino, donde la fase estacionaria presenta alta área superficial de compuestos paramagnéticos (SiO_2/Fe_3O_4) y una intensidad de campo variables de 0 a 5,5 mT retiene sobre ella sustancias paramagnéticas[294].

En menor medida, se ha empleado la resonancia magnética nuclear en matrices de vino[295], para establecer huellas metabólicas en diferentes vinos y procesos de vinificación[296-299], o para la caracterización de autenticidad del vino, origen geográfico, y año de producción a través del perfil conjunto de carbohidratos, AcOrg y AAs[300].

La electroforesis capilar (CE) ha sido reportada en la separación y detección de AAs, péptidos y proteínas en vino cuando se acopla a distintos detectores[301,302]. Los detectores más empleados acoplados a CE son conductividad y UV-Visible (185-254 nm) o conjuntamente[303]. Sin embargo, la principal desventaja de esta metodología es el procedimiento de preparación de muestra, ya que requiere de una derivatización y precipitación previa al análisis[295].

1.8.3 CARBOHIDRATOS

Existen referencias bibliográficas del empleo TLC en el estudio del perfil de azúcares en diferentes tejidos vegetales de *Vitis vinifera* como hojas, raíces, corteza, frutos y madera[220]. Por otro lado, la cromatografía de gases ha sido también ampliamente utilizada para la determinación de carbohidratos en tejidos vegetales de hojas de *Vitis vinifera*[304,305] o en la pulpa y piel de las bayas[268]. No obstante, esta última requiere una derivatización a través de sus derivados de alquil-éteres, alquil-ésteres o la trimetilsililados[306,307], y comúnmente se utiliza acoplada a detectores FID y MS[308,309].

Sin embargo, la técnica más destacada para la determinación de azúcares es LC, sobre todo, SEC, la cromatografía quiral[310,311], HILIC o IEC[312-314]. Los detectores más utilizados con HPLC son RID[315,316], que permite la determinación de derivados fosfatados, ELSD[291,317], DAD para el estudio de isómeros D y L[311] o detectores electroquímicos[318]. De hecho, la OIVV recomienda un método HPLC acoplado a un detector RID para la determinación de azúcares en mosto y vino[319]. Este método ha sido de gran aplicación en el análisis simultáneo de azúcares, AAs y AcOrg[256,320,321].

La técnica RMN ha sido empleada, en menor medida, para la identificación de carbohidratos simples y/o complejos incorporados en matrices de alimentos[322], o en la determinación de carbohidratos acumulados en tejidos vegetales durante el estrés por frío[323].

La electroforesis capilar ha permitido la determinación directa e indirecta de carbohidratos gracias a agentes derivatizantes como 6-aminoquinolona o ácido 4-aminobenzoico[324,325]. Por lo que también tiene aplicación en el análisis de carbohidratos en hojas de vid[326] o en vino tinto y blanco[302] mediante el acoplamiento a espectrometría de masas.

Chapter II: Objectives

2.1 OBJECTIVES

As a result of the climatic changes happening during the last decades, a temperature intensification and the increase of drought periods are expected in many areas of the Rioja wine-growing region (Spain). For that, *Vitis vinifera* has suffered some disarrays in their reproductive and vegetative growth cycles, just like in physiological and biochemical behavior, leading to weakening in the yield and quality of the fruits.

In this respect, there is a need for more profound research to find new, eco-friendly agricultural practices that improve the availability of water and nutrients in the soil and their uptake by the plant. In this context, the application of biostimulants represents one of the most promising tools for minimizing stress impact in many crops, including *Vitis vinifera*. However, only a few studies report the effect of these substances in grapevine plants or determine their mechanism and mode of action, mainly performed under the climate change scenario.

For this reason, the main objectives of this work are the following:

- Develop an experimental field plan for studying the effect of foliar application of three different commercial biostimulant products, based on seaweed extracts and vegetable amino acids, in old and low productive plants of *Vitis vinifera* variety "Tempranillo" located in a semi-arid region of Spain (La Rioja).

- Determine and quantify organic acids, free amino acids, and carbohydrates in different tissues of *Vitis vinifera* using targeted metabolomics. They will be based on liquid chromatography (HPLC) coupled to several detectors as Refractive Index Detector (RID), Diode Array Detector (DAD), and Fluorescence Detector (FLD) to obtain the highest sensitivity and the lowest measurement uncertainty, ensuring the quality of the data obtained.

- Determine the mechanism and mode of action of three biostimulants based on seaweed or plant-derived amino acids using the multi-trait high-performance screening (MTHTSM) method using *Arabidopsis thaliana* (accession Col-0) as a model plant grown under water stress conditions.

- Evaluate the biological translation from *Vitis vinifera* to *Arabidopsis thaliana* regarding the biostimulant-induced response in the plants grown under water stress conditions.

Chapter III: Materials and Methods

3.1 EXPERIMENTAL CONDITIONS FOR *VITIS VINÍFERA* L. (CV. TEMPRANILLO)

3.1.1 PLANT MATERIAL AND GROWTH CONDITIONS

This work was carried out in a vineyard belonging to the DOCa Rioja, located in Cenicero [42°27' North Latitude, 2°39' West Longitude, and an altitude of 436 m, La Rioja (Spain)]. The climate is typically Atlantic, characterized by being warm with significant rainfall, even during the driest months. For this reason, the vines are exposed to harmful external factors such as frosts, hail, or strong winds that might cause grape quality and harvest loss. The vineyard comprises 2,230 m^2 of red cv. Tempranillo vines variety (*Vitis vinifera* L.), grafted on 110 Richter rootstocks. In the experiment plot, vines were planted in 1984 and trained on a regular clay and sandy soil frame under rainfed conditions in 9 traditional rows in a training cup with 3,365 vines/ha. During the vegetative cycle, agricultural practices were carried out under the designation of Corporation DOCa Rioja. For example, Circular 14/2004 regulated the pruning and established a maximum load per vine of 12 buds on a maximum of 6 thumbs, limited to 6,500 Kg/ha[327].

The study was performed for two consecutive years (2018 and 2019). The vineyard suffered incidences recorded in the field notebook during both seasons and detailed in **Annex I**, *Table I.1, Table I.2,* and *Fig. I.1-I.10.* The DOCa Rioja recommended the use of phytosanitary products against mildew, botrytis, oidium, powdery mildew, and/or mites were required for supporting the vineyard healthiness. In concrete, during the veraison of both seasons, the vineyard suffered severe damage by intense storms and hail, causing the grape grains to breakage and bunches to decay in some treatments. In addition, this damage caused low-quality grape berries and diseases such as mildew, oidium, and powdery mildew. Likewise, during the 2019 vintage, the vineyard suffered slight damage caused by erinosis, fungal infections induced by *Tereum hirsutum* Per. and *Phellinus igniarius* Fr.), and the attack of leafhoppers (*Empoasca vitis*).

3.1.2 EXPERIMENTAL DESIGN

Three commercial biostimulants were applied using foliar application following the producers' recommendations to improve the quality and quantity of the grapevine production. The three products have similar origins; Basofoliar Avant Natur (BF, Martinez Carra S.L., Calahorra, La Rioja) based on 100% plant extracts rich in amino acids, and SoilExpert (ST, Alfa & Omega Consulting S.L., Puente La Reina, Navarra) and Greetnal (GT, Lainco S.A., Barcelona) both based on seaweed AN extract. Two additional treatments were included a negative control (C) using water at the same volume and a positive control applying the non-proteic amino acid BABA. The foliar applications were made in two consecutive vintages, corresponding to 2018 and 2019, in which two foliar applications were made, the first foliar application before flowering (May) and the second one after flowering (June). 3 L ha^{-1} of BF and 2.5 L ha^{-1} of ST and GT were used in each application as recommended by the manufacturer. The amino acid BABA was applied at 0.1 mM.

For the experiment, the vineyard was divided into two subplots[328], **A** facing Northwest and **B** at Southeast orientation. In both, five intercropped rows were selected to achieve randomly distributed foliar applications as described in *Fig. 3.1*.

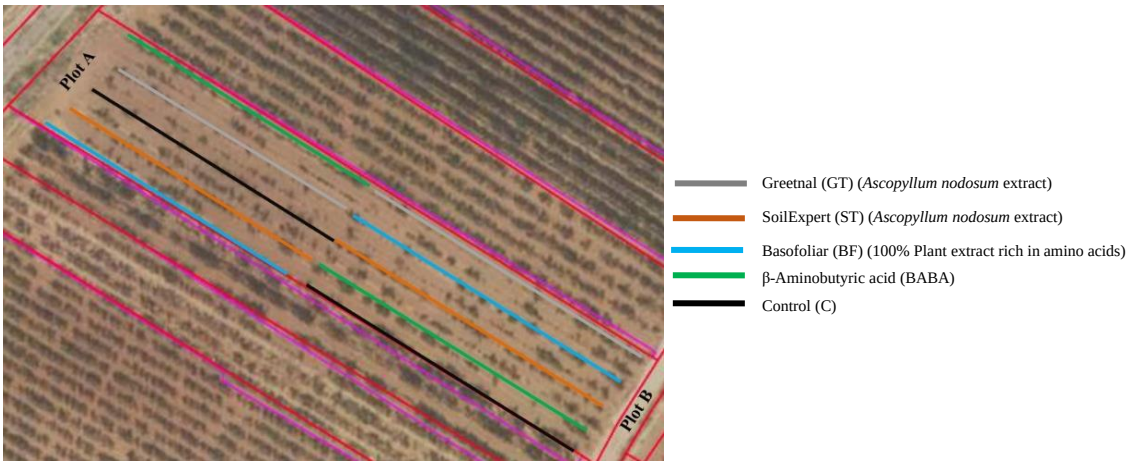


Figure 3.1. *Experimental design of foliar applications in the vineyard. It was adapted from "https://www.sigpac.mapa.gob.es"* [329].

3.1.3 SAMPLING AND SAMPLE STORAGE

Leaves and fruits from *Vitis vinifera* L. were selected as target matrices to develop this work. Their collection, storage, and procedures for quantification are described below.

3.1.3.1 LEAVES

Five vines (one every 3 strains) with similar developmental stages per row and plot were selected for the study (*Fig. 3.2a*). High or low topographic positions and vigorous or weak strains were avoided to compare more homogeneous samples. The samples were collected in the early hours of the day to prevent enzymatic degradation of the plant material.

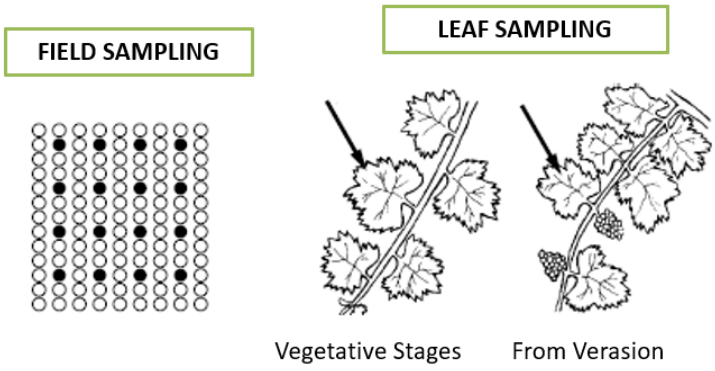


Figure 3.2. *a) Field sampling plan and labeled vines for homogeneous sampling within the vineyard. b) Leaf and fruits sampling methodology according to the phenological stage. Author: https://www.agroconciencia.com.ar/fvides.pdf and http://spectrumanalytic.com*[330,331].

Five samplings throughout the life vine cycle were made; two before and 24 h after the first foliar application before the flowering (May) defined as T0A and T24A, respectively, other two before and 24 h after the second foliar application after the flowering defined as T0B and T24B, respectively, where the samples are on the same side and closer to the bunch[330].

The last sampling, TF, were carried out during the harvest (October). From veraison, it is recommended to collect the leaf opposite basal fruit cluster[330] (*Fig. 3.2b*). All collected samples were cut, homogenized, and stored frozen at -40°C until their analysis (*Fig. 3.3)*.

Figure 3.3. *Transport, homogenization, and storage of the collected* Vitis vinifera *L. leaf samples.*

3.1.3.2 MUST AND GRAPE PASTE

Vitis vinifera fruits were collected twice; before the harvest (September, TV) and after the harvest (October, TF) when the fruit was fully ripped. Before the harvest, the basal and consecutive bunches were collected. The other two bunches were also harvested from the remaining brunches during the ripening.

Grape bunches were removed from their stems, homogenized, and separated into two aliquots. The first aliquot, destined to must, was squeezed using a mortar and filtered to obtain the pulp juice. The second aliquot, destined for grape paste, was crushed with a domestic blender with seeds and skins (*Fig. 3.4*). Then, the samples were frozen at -40°C until their analysis.

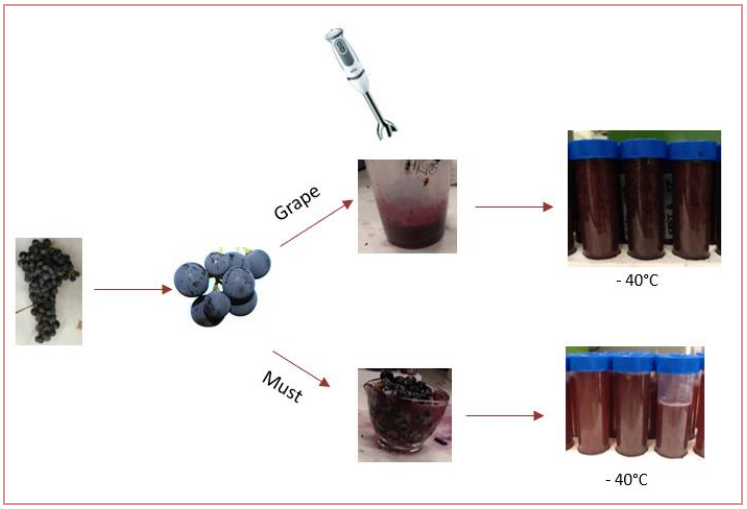


Figure 3.4. *Transport, homogenization, and storage of* Vitis vinifera *L. fruit samples for preparing must and grape paste.*

3.1.4 WATER STATUS DETERMINATION

Leaf water content (LWC, %) was determined in two vintages collected from T0B and T24B leaves of *Vitis vinifera* L., as follow:

$$\text{LWC}\ (\%) = \frac{\text{FW} - \text{DW}}{\text{DW}} \cdot 100 \qquad \textit{(Eq 3.1)}$$

Where FW is the fresh weight of the leaf and DW is the dry weight of the leaf after 48 h of drying in the oven at 60ºC.

3.1.5 *VITIS VINIFERA* L. CROP PRODUCTION PARAMETERS

3.1.5.1 YIELD PARAMETERS AND SURVIVAL

Yield parameters were calculated at maturity (TV) and harvest (TF) by assessing vine yield cluster (kg/vine) and cluster number per vine (n). Moreover, the average cluster weight (g), length (cm), and berry diameter (mm) were studied to determine the effect of biostimulants application.

After the dormancy, the vine's survival (SV, %) for each variant was estimated using the *Eq. 3.2*[332]:

$$SV(\%) = \frac{PV}{(PV + PM)} \cdot 100 \qquad \textit{(Eq 3.2)}$$

Where PV is the number of survivors and PM is the number of dead plants.

3.1.5.2 CLIMATE DATA, PHENOLOGICAL STAGES AND WATER USE EFFICIENCY

During the 2018 and 2019 seasons, the climatic conditions were recorded in meteorological stations placed nearby the analyzed plots in Cenicero. The information included daily maximum temperature, daily average temperature, daily minimum temperature, and precipitation. In **Annex II** *(Table II.1),* the monthly data of maximum temperature (Tmax., °C) and minimum temperature (Tmin., °C), the average temperature (Tavg., °C), and precipitation (P, mm) are detailed.

The reference crop evapotranspiration (ET_0, mm) was estimated as proposed by Hargreaves and Samani in 1985[333]:

$$ET_0 = (0.0023) \cdot (T_{avg} + 17.8) \cdot (T_{max} - T_{min})^{0.5} \cdot R_a \qquad \textit{(Eq 3.3)}$$

Where R_a is the extraterresterial solar radiation (mm day^{-1}).

Then, effective crop evapotranspiration (ET_c, mm) was determined. This term corrects the deficiencies of the previous equation by a K_c factor that depends on the moisture soil level, crop characteristics, and the stage of the crop vegetative cycle[51]. For vines whose fruits are destined for wine production and with a maximum height between 1.5-2 m, K_c values are estimated at 0.3 in the initial phases of the cycle, 0.7 in the intermediate phases, and 0.45 in the final stages of the vegetative cycle[334].

$$ET_c = ET_0 \cdot K_c \qquad \textit{(Eq 3.4)}$$

Water deficits (WD) were quantified as accumulated precipitation minus crop evapotranspiration. Water deficits were determined during growing seasons (1st March-30th September); Budbreak (BB), Bloom (BL), Veraison (V), and Maturity (M) for periods between phenological stages; BB-BL, BL-V, and V-M[51]. Additionally, the budding dates during the growing seasons, BB, Bl, V, and

M, are detailed in **Annex II** *(Fig. II.1).* The beginning of the growing season (BB) was determined by direct observation and daily monitoring of the vineyard (*Table II.2).*

In addition, production water use efficiency (WUE, kg mm^{-1}) of the vines was also estimated based on Kiziloglu *et al.* (2006)[335]. The accumulated ET_c from budbreak to maturity was calculated. The water use efficiency was then calculated as the ratio between the accumulated ET_c and the final yield (kg ha^{-1}).

3.1.6 OSMOLYTES QUANTIFICATION

Special consideration must be given to the quantitative determination of endogenous compounds such as the analytes of interest in this study in the proposed matrices. Matrix effect (ME) is the main drawback in quantifying and validating analytical methodologies of endogenous compounds. Calibration standards must be performed in the same matrix as the study samples, and the analyte-free matrix is often unavailable. For this reason, the reproducibility, linearity, and precision method are limited to the nature of the sample[336,337].

In this work, an analyte-free matrix was impossible to quantify endogenous compounds. Once considering all the advantages and inconveniences of the methods described in literature[338,339], an external calibration method based on mobile phase solvents or pure water was proposed as a surrogate matrix to quantify primary metabolites in leaf and fruit of *Vitis vinifera* L. To achieve this requirement, the recovery of the extraction procedure and ME should be evaluated. Some studies reported the matrix effect evaluation based on the analytical methodologies used[340-350].

According to the European Commision[351], a representative matrix mixture is needed for the standard addition method. In this work, a representative pooled leaf, must, or grape taken from all treatments carried out in the vineyard were considered as blank control (C) and the same extraction procedure before the HPLC analysis was made. The ME was studied according to Gonzalez-Gonzalez *et al.* (2019)[352] and evaluated the homogeneity of variance using F-test (Snedecor) and compared the means of both quantification methods using Student's t-test[353] is necessary. In Student's t-test, means of both methods were statistically compared by the value of t_{Exp} (*Eq. 3.5*) and t_{Crit} (α, n_1+n_2-2), where n_1 *and* n_2 are the numbers of levels used to build the respective calibration curves. The t'_{Crit} value of non-homogeneous variance is calculated using *Eq. 3.6*.

$$t_{Exp} = \frac{|b_1 - b_2|}{\sqrt{S_{b1}^2 + S_{b2}^2}} \cdot 100 \qquad \textit{(Eq 3.5)}$$

$$t'_{Crit} = \frac{t'_1 b_1 + t'_2 . b_2}{S_{b1}^2 + S_{b2}^2} \cdot 100 \qquad \textit{(Eq 3.6)}$$

Where t'_1 (α, n_1-2) and t'2 (α, n_2-2).

There are several accepted patterns regarding the determination of the limits of quantification (LOQs). In this study, the quantification limits were estimated as the minimum amount of analyte in a sample that can be determined with an acceptable level of accuracy and precision, considering the variation coefficient lower than 20%[354].

Precision is the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed analytical conditions[355]. Precision is calculated in terms of repeatability and intermediate precision after analyzing five replicates (n=5) during five consecutive days, both calculated and expressed as relative standard deviation (RSD).

In this study, certified reference material is not available. The accuracy was calculated in terms of recovery at two different fortified levels[356,357]. The recovery of the extraction method will be studied as[358]:

$$Recovery\ (\%) = \frac{C_f - C_u}{C_a} \cdot 100 \qquad \textit{(Eq 3.7)}$$

Where C_f and C_u correspond to fortified and unfortified test samples and C_a is the concentration added to the sample.

3.1.6.1 CARBOHYDRATES QUANTIFICATION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO REFRACTIVE INDEX DETECTOR (HPLC-RID)

3.1.6.1.1 MATERIALS

3.1.6.1.1.1 STANDARDS AND REAGENTS

Analytical standards were obtained from different manufacturers. D-(-)-fructose (Fruc) from Sigma-Aldrich (Steinheim, Germany), D-(+)-glucose (Gluc) and sucrose (Sac) from Panreac (Barcelona, Spain), and maltose monohydrate (Malt) from Merck (Darmstadt, Germany). All of them with analytical quality >99%.

All organic solvents, acetonitrile (ACN), chloroform ($CHCl_3$), and methanol (MeOH), were spectrally pure for LC-MS, and were supplied by Scharlab (Barcelona, Spain). Ultra-high purity water, obtained from tap water pre-treated by Elix20 reverse osmosis before filtration by a Milli-Q system from Millipore (Bedford, MA, USA) was used throughout the study.

3.1.6.1.1.2 PREPARATION OF STANDARD SOLUTIONS

Stock standard solutions of each carbohydrate-containing around 50,000 mg L^{-1} and 5,000 mg L^{-1} were prepared in ultra-high purity water. From these solutions, intermediate mixed solutions of 100 mg L^{-1}, 150 mg L^{-1}, and 200 mg L^{-1} were prepared in the mobile phase at initial conditions. All these solutions were stored in darkness at 4°C.

Calibration standards were prepared in the mobile phase at initial conditions in a range of concentration from 150 mg L^{-1} to 5,000 mg L^{-1} of all analytes. These solutions were also used to calculate the LOQs of the analytical method. Calibration curves were built at different ranges depending on the compound and the matrix of *Vitis vinifera* L. used.

There is no analyte-free matrix available for endogenous carbohydrates determination and the same modified matrix, so it is impossible to build the calibration curves. A previous standard addition calibration method was necessary to quantify pooled leaves, must, and grapes beforehand. Pooled

leaves, musts, and grapes were spiked with increasing amounts of carbohydrates to produce different concentrations depending on the compound and matrix. The initial standard solution to spike pooled leaves contained 4,350 mg L^{-1} of Fruc, 4,345 mg L^{-1} of Gluc, 880 mg L^{-1} of Sac and 4,783 mg L^{-1} of Malt. Pooled musts and grapes were spiked with a solution containing 42,080 mg L^{-1} of Fruc and 32,380 mg L^{-1} of Gluc.

3.1.6.1.2 METHODS

3.1.6.1.2.1 EXTRACTION METHOD PROCEDURE

One-hundred leaf samples, forty must and forty grape samples of *Vitis vinifera* L. (cv. Tempranillo) previously stored at -40°C were analyzed. All measurements were made in triplicate.

- **LEAVES**

From leave matrix, Fruc, Gluc, Sac and Malt were extracted according to a previously described method[355,359]. 250 µL of ice-cold chloroform blended with methanol (3:7, % v/v) was added to 10 mg of dry weight samples and vortex for 30 s. Then, samples were incubated at -20°C for 2 h with occasional vortex-mixing. 200 µL of ice-cold water was added to the mixture, and the samples were vortex for 3 min until they were tempered. Then, the samples were centrifuged (10,000 rpm) at 4°C for 10 min using a 5415R Eppendorf™ microcentrifuge from Marshall Scientific (Hampton, USA). The aqueous-methanol phase was transferred to new 1.5 mL centrifuge tubes. As described above, the chloroform phase was re-extracted with 200 µL of ice-cold water. The aqueous-methanol phases were mixed and evaporated to dryness under a gentle nitrogen stream.

The dry residues were reconstituted in 250 µL of ACN/H_2O (80:20). Finally, all the samples were centrifuged at 8,000 rpm for 5 min and filtered through 0.22 µm polyvinylidene fluoride (PVDF) syringe filters from (Tecknokroma, Barcelona, Spain), as shown in *Fig. 3.5.*

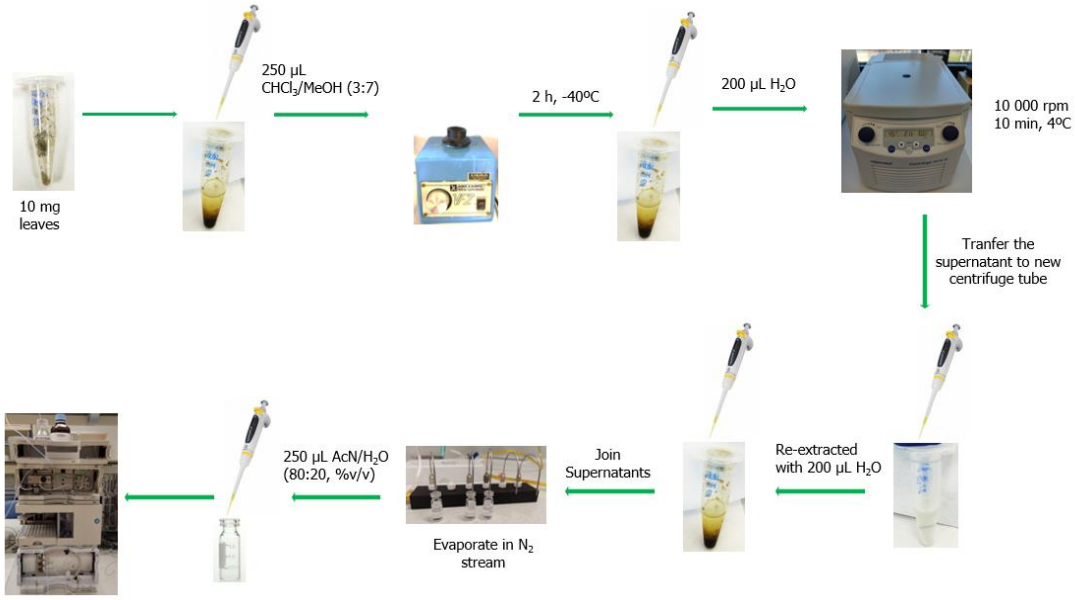


Figure 3.5. *Sample treatment for carbohydrates extraction in leaves.*

- **MUST AND GRAPES**

For carbohydrates determination in grape samples, 200 µL of ice-cold water was added to 100 mg of the homogenized material. The mixtures were shaken for 5 min and centrifuged at 10,000 rpm at 4°C for 10 min using a 5415R Eppendorf™ microcentrifuge. The extracts were diluted at 1:20 in ACN/H_2O (80:20) and centrifuged at 8,000 rpm for 5 min.

In musts, 980 µL of ice-cold water was added to 20 µL of homogenized samples. Then, the mixtures were shaken for 5 min and centrifuged at 10,000 rpm at 4°C for 10 min. All measurements were filtered through 0.22 µm polyvinylidene fluoride (PVDF) syringe filters prior to injection of 5 µL to HPLC analysis.

- **BIOSTIMULANTS**

Direct injection dilutions into the high-performance liquid chromatography coupled to a refractive index detector (HPLC-RID) system determined the carbohydrate content in ST, GT, and BF.

3.1.6.1.2.2 INSTRUMENTAL ANALYSIS

HILIC-RID analyses were carried out on an Agilent 1100 series HPLC system supplied with Agilent 1100 series quaternary pump and Agilent 1260 degasser coupled to a refractive index detector (RID) Agilent 1260 from Agilent Technologies (Santa Clara, CA, USA) operating at 45°C.

Chromatographic separation was performed on a ZIC®-HILIC SeQuant® (3.5 µm, 150 x 4.6 mm i.d.; Merck, Darmstadt, Germany) protected with ZIC®-HILIC SeQuant® guard column (5 µm, 20 x 2.1 mm i.d.; Merck, Darmstadt, Germany) keeping at 60°C during the run. The flow rate was 0.7 mL min^{-1}, and the sample injection volume was 5 µL. The mobile phase was composed of ACN/H_2O (80:20, %v/v), and the LC run time was 20 min using an isocratic elution, followed by column re-equilibration of 5 min. The mobile phases were filtered through 0.22 µm Polytetrafluoroethylene (PTFE) filters from Millipore (Watford, Ireland) and degassed with a Digital Ultrasonic Cleaner CD-4820 (Medellín, Colombia) ultrasonic system. Agilent LC ChemStation software (Agilent Technologies, Santa Clara, CA, USA) was used for system control and data analysis.

Leaf samples were lyophilized with a LyoQuest 85/230V, 50 Hz from Telstar (Tokyo, Japan) and evaporated in a Dry-Block® DB-3D Techne evaporator (Staffordshire, UK). Before the injection into the HPLC system, all the samples were centrifuged in a 5415 R Eppendorf™ microcentrifuge from Marshall Scientific (Hampton, USA).

3.1.6.1.2.3 MATRIX EFFECT EVALUATION

The ME was expressed as the relation between the slopes obtained by external calibration and the standard addition method. Each concentration level was analyzed in triplicate.

As shown in **Annex III** (*Table III.1-III.3*) the ME is always within ±5%. Likewise, the results obtained by means of standard addition and standards prepared in water were statistically comparable (F_{Exp}=1.04-4.78<$F_{Critical}$=4.32-9.24, and T_{Exp}=0.01-0.73<T_{Crit}=1.86-1.94) at 95% of confidence level. For this reason, the external calibration method was considered an excellent tool for carbohydrate quantification by HPLC-RID in leaves, musts, and grapes because no matrix

effects were detected. Likewise, *Fig. III.1-III.3* shows the graphic comparison between both quantification methods used for leaf, must, and grape samples of *Vitis vinifera* L.

3.1.6.1.2.4 ANALYTICAL METHOD EVALUATION

External calibration curves in water were prepared at different concentrations depending on the compound. These equations were obtained facing analyte signal with abundances from chromatograms areas from retention index detector. Regression equations of target compounds are summarized in *Tables 3.1* and *Table 3.2*. The correlation coefficients above 0.9991 were achieved for all analytes. In order to verify if linear regression models proposed for each compound are acceptable, a second statistical analysis was necessary on the random distribution of the residues. This assumption must meet that if the data distribution is normal, the arithmetic means of the residues, $\bar{r}$, is expected to be close to 0. In addition, the standard deviation of the residues σ(r), was equal or less than the global standard deviation, σ_{TOT}, and average residue, $|\bar{r}|$, was equal to or lower than σ(r). The statistical analysis results on the random distribution of the residues shown in *Table 3.1* and *Table 3.2* verify that the requirements to assert the proposed linear regression equations are adequate to obtain data.

Table 3.1. *Calibration curves and statistical analysis on the random distribution of the residues quantifying carbohydrates in leaf samples of Vitis vinifera L.*

Analytes	Calibration Range (mg L^{-1})	Slope ± error	Intercept ± error	Correlation coefficient (r)	σ_{TOT}	$\bar{r}$	σ(r)	$\lvert\bar{r}\rvert$
Fruc	154.00-2,570.00	70.64 ± 1.19	264.07 ± 1,714.12	0.9996	2,256.82	0.00	1,748.12	1,525.53
Gluc	155.00-5,150.00	32.02 ± 0.28	584.82 ± 696.81	0.9998	1,153.04	0.00	941.45	789.82
Sac	155.00-3,040.00	64.57 ± 0.43	-1,584.83 ± 676.10	0.9999	990.23	0.00	767.03	528.69
Malt	146.00-2,430.00	17.24 ± 0.12	169.20 ± 155.29	0.9998	241.10	0.00	196.85	184.82

Table 3.2. *Calibration curves and statistical analysis on the random distribution of the residues quantifying carbohydrates in must and grape samples of* Vitis vinifera *L.*

Analytes	Calibration Range (mg L^{-1})	Slope ± error	Intercept ± error	Correlation coefficient (r)	σ_{TOT}	$\bar{r}$	σ(r)	$\lvert\bar{r}\rvert$
Fruc	154.00 -2,570.00	70.64 ± 1.20	264.07 ± 1,714.12	0.9991	2,256.82	0.00	1,784.12	1,525.53
Gluc	158.00-5,150.00	31.66 ± 0.33	1,434.34 ± 482.42	0.9997	635.15	0.00	492.00	436.10

In addition, for each analyte, the limits of quantification were 154.00 mg L^{-1} for Fruc, 155.00 mg L^{-1} for Gluc, 155.00 mg L^{-1} for Sac, and 146.00 mg L^{-1} for Malt.

The precision of the developed method was calculated for low, medium, and high standard calibration levels in the proposed calibration curves. The repeatability and intermediate precision were also determined after analyzing five replicates ($n=5$) during five consecutive days, which everyone calculated and expressed as relative standard deviation (RSD). As shown in *Table 3.3*, the method precision was within the acceptable range in this study. The repeatability of measurements was between 0.94-10.07%, and the intermediate precision among 3.28-11.38% for all the analytes.

Table 3.3. *Summary of validation parameters of HPLC-RID analytical method.*

	LOQ (mg L^{-1})	Repeteability (%RSD)			Intermediate Precision (%RSD)		
		Low level	Medium Level	High Level	Low level	Medium Level	High Level
Fruc	154.00	9.81	5.89	2.27	8.58	8.22	3.28
Gluc	155.00	5.49	10.07	3.36	10.97	11.38	6.42
Sac	158.00	3.07	7.00	5.37	6.34	8.89	5.04
Malt	146.00	4.44	3.02	0.94	7.25	8.96	8.49

Finally, the efficiency of the sample preparation procedure was evaluated through recovery assessment. The addition of standard solutions in the pooled leaves and grapes before the extraction process at two concentration levels in QC samples was used to evaluate the recovery of the extraction process, analyzing five replicates ($n=5$) per level.

Table 3.4 and *Table 3.5* shows the recoveries within 80.23-101.32% and 90.47-100.81% range for leaf and grape matrix, respectively. Just like that, it can be concluded that the respective developed

sample treatments procedures provide an acceptable recovery for the analytes studied in V*itis vinifera* L. leaves and grapes.

Table 3.4. *Recovery (%) and standard deviation of carbohydrates in a pooled leaf of* Vitis vinifera *L.*

Recovery (%)	Low level	High level
Fruc	80.23 ± 7.00	101.32 ± 1.70
Gluc	97.46 ± 5.97	101.16 ± 4.82
Sac	83.98 ± 15.13	90.12 ± 3.87
Malt	100.21 ± 4.42	98.20 ± 5.10

Table 3.5. *Recovery (%) and standard deviation of carbohydrates in a pooled grape of* Vitis vinifera *L.*

Recovery (%)	Low level	High level
Fruc	90.47 ± 7.79	100.06 ± 7.17
Gluc	98.81± 5.41	100.81 ± 5.52

3.1.6.2 ORGANIC ACIDS QUANTIFICATION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO PHOTODIODE ARRAY DETECTOR (HPLC-DAD)

3.1.6.2.1 MATERIALS

3.1.6.2.1.1 STANDARDS AND REAGENTS

The analytical standards were obtained from two different manufacturers. Anhydrous oxalic acid (Oxal, >98%), L-(+)-tartaric acid (Tart, >99%), DL-malic acid (Mal, >99%), acetic acid (Acet, >99.5%), citric acid (Cit, >98%), succinic acid (Succ, >99%) and fumaric acid (Fum, >99%) from Cymit Química (Barcelona, Spain).

All the solvents for the mobile phase were spectrally pure for LC-MS, and the reagents were of analytical quality (>98%). For the mobile phase and standard preparation, sodium biphosphate

monohydrate ($NaH_2PO_4 \cdot H_2O$) and 85% phosphoric acid (H_3PO_4) from Merck (Darmstadt, Germany), acetonitrile (ACN) from Scharlab (Barcelona, Spain) were acquired.

For the extraction of organic acids in leaves, sodium hydroxide (NaOH) from Merck (Darmstadt, Germany) and sodium chloride (NaCl) from Panreac (Barcelona, Spain) were used. Ultra-high purity water was obtained from tap water pre-treated by Elix20 reverse osmosis and purified by Milli-Q filtration system from Millipore (Bedford, USA).

3.1.6.2.1.2 PREPARATION OF STANDARD SOLUTIONS

Stock standard solutions 1,000 mg L^{-1} for each organic acid were prepared in ultra-high purity water, except for Oxal, Tart, and Mal, which were added at 10,000 mg L^{-1}. From these solutions, individual standard dilutions of 100 mg L^{-1} were prepared. To determine the limits of quantification, mixed solutions containing 0.005 mg L^{-1}, 0.01 mg L^{-1}, 0.05 mg L^{-1}, 0.1 mg L^{-1}, 0.25 mg L^{-1}, 0.5 mg L^{-1} and 1 mg L^{-1} were prepared. These solutions were prepared in ultra-high purity water and stored in darkness at 4°C.

Calibration standards were prepared in ultra-high purity water and contained between 0.025-1,065 mg L^{-1} for Oxal, 0.25-2,090.00 mg L^{-1} for Tart, 0.25-2,072.00 mg L^{-1} for Mal, 1.00-1,035.00 mg L^{-1} for Acet, 0.50-564.00 mg L^{-1} for Cit, 0.50-100.86 mg L^{-1} for Succ and 0.01-2.75 mg L^{-1} for Fum. Calibration curves were built at different ranges depending on the compound and the plant material used. The degradation of working solutions induced the need to prepare new ones weekly.

As there is no analyte-free matrix available for endogenous organic acids, a previous standard addition calibration method was necessary to quantify pooled leaves, must, and grape beforehand. For that purpose, pooled leaves, musts, and grapes were spiked with increasing amounts of organic acids to produce different concentrations depending on the compound and the plant material. Pooled leaf was spiked with other prepared mixed standard solution containing 12,270 mg L^{-1} of Oxal, 100,250 mg L^{-1} of Tart, 69,600 mg L^{-1} of Mal, 24,780 mg L^{-1} of Acet, 5,890 mg L^{-1} of Cit, 675 mg L^{-1} of Succ and 5.03 mg L^{-1} of Fum. A pooled must was spiked with a solution containing 10,923 mg L^{-1} of Oxal, 29,163 mg L^{-1} of Tart, 22,090 mg L^{-1} of Mal, 2,190 mg L^{-1} of Acet, 5,560 mg L^{-1} of Cit, 703 mg L^{-1} of Succ and 26.5 mg L^{-1} of Fum. A pooled grape was spiked with a solution containing 16,960 mg L^{-1} of Oxal, 60,490 mg L^{-1} of Tart, 28,430 mg L^{-1} of Mal, 2,625 mg L^{-1} of

Acet, 6,190 mg L^{-1} of Cit, 450 mg L^{-1} of Succ and 13.6 mg L^{-1} of Fum. These solutions were prepared by joint weighing.

3.1.6.2.2 METHODS

3.1.6.2.2.1 EXTRACTION METHOD PROCEDURE

One hundred leaf samples, forty must, and grape samples of *Vitis vinifera* L. (cv. Tempranillo) were analyzed in the present work. All measurements were made in triplicate.

- **LEAVES**

Extraction was achieved as previously reported Hazer *et al.* (2016)[360], with some minor modifications. Briefly, 5 g of each fresh sample was mixed with 20 mL of 0.1 mM sodium hydroxide, and the mixture was stored in darkness at 4°C for 24 h. Samples were ultrasonicated in a Digital Ultrasonic Cleaner CD-4820 from JeKen (Dongguan, Guangdong, China) for 30 min, and the mixture was filtered with Whatman™ paper filters (55 mm ø). Then, leaf samples were washed with 5 mL of sodium chloride (5% w/v). After shaking samples for 5 min, the mixture was rinsed twice with 1.5 mL of Milli-Q water to ensure total extraction of organic acids and acidified to pH 2.7 with H_3PO_4 (50% v/v). Finally, samples were centrifuged in an Allegra™ X-22R centrifuge from Beckman Coulter (Brea, USA).

- **MUST AND GRAPES**

Organic acids were extracted in grapes with water, as previously reported Flores *et al.* (2012)[254]. Briefly, 3 g of samples were homogenized with 10 mL of MiliQ water. The mixture was centrifuged at 10,000 rpm at 4°C for 10 min using a 5415R Eppendorf™ microcentrifuge from Marshall Scientific (Hampton, USA) and diluted at 1:2 with the mobile phase solution at initial conditions.

Homogenized must sample was centrifuged at 10,000 rpm at 4°C for 15 min and diluted at 1:3 with mobile phase solution at initial conditions[244]. Before performing the respective dilutions, each sample was adjusted to pH 2.7 with H_3PO_4. Finally, all extracts were filtered through 0.22 µm PVDF syringe filters prior to the injection of 20 µL to HPLC analysis.

- **BIOSTIMULANTS**

To characterize the biostimulants, an aliquot of ST, GT and BF was diluted at 1:200 in the mobile phase at initial conditions and filtered through 0.22 µm PVDF syringe filters before injection in the HPLC system.

3.1.6.2.2.2 INSTRUMENTAL ANALYSIS

Chromatographic analyses were carried out on an Agilent 1290 infinity series HPLC system (Agilent Technologies©, Palo Alto, California, USA) equipped with an automatic liquid sampler (Agilent Technologies©, 1290 infinity), a column thermo-regulator, and a photodiode array detector (DAD) (Agilent 1290 infinity). The chromatographic separation was carried in an analytical UltraAqueous C-18 column (4.6 id x 150mm, 5µm) from Waters Corporation (Milford, MA, USA) thermostated at 25°C.

The mobile phases were (A) 99:1 NaH_2PO_4/ACN solution (4mM, pH 2.7, with H_3PO_4) and (B) was ACN. Mobile phases were filtered through the Millipore Omnipore from Millipore (Watford, Ireland) 0.1 µm filters. The pH of mobile phases and samples was measured with GLP21 Crison pHmeter (Alella, Spain). 20 µL of the sample was injected and analyzed in a mobile phase gradient with a flow rate of 0.5 mL min^{-1}. The elution was programmed with 0% B for 4.9 min, 0–20% B for 7.1 min, 20–60% B for 1 min, 60% B for 2 min, 60–0% B for 2.5 min, and 0% B for 2.5 min followed by column re-equilibration in A for 5 min. Diode Array detection was set a 210 nm. ChemStation Rev. B.04.02 software (Agilent Technologies©, Palo Alto, California, USA) was used for data acquisition, peak integration, and standard calibration.

3.1.6.2.2.3 MATRIX EFFECT EVALUATION

Due to the higher number of samples, external calibration as a surrogate matrix was an alternative to obtaining a free-analytes matrix. For this reason, the ME in all matrices from *Vitis vinifera* L. was evaluated as previously described (section 3.1.6). Each concentration level in standard addition and external calibration was carried out in triplicate ($n=3$), and the relative standard deviations (RSDs) in each level were less than 5% for all analytes studied. Statistical comparison at 95% of the

confidence level of the matrix effect of preparing analytical standards in water and standard addition method was performed.

As shown in **Annex IV** (*Table IV.1-IV.3*), the ME is always within ± 5% for all the analytes studied. On the one hand, the F-test study, homogeneous variances for citric, succinic, and fumaric acids in leaves (F_{Exp}=0.31-2.97<F_{Crit}=2.20-5.46), for Oxal, Acet, Cit and Succ in grapes (F_{Exp}=1.22-4.79<F_{Crit}=4.32-9.24) and for Acet, Succ and Fum in musts (F_{Exp}=0.94-3.40<F_{Crit}=3.78-5.46) has been verified. For these instances, the Student t-test is statistically comparable (T_{Exp}=0.03-1.59<T_{Crit}=1.83-2.37) at 95% confidence level for all the analytes in each matrix *Vitis Vinifera* L. On the other hand, non-homogeneous variances had been observed in Oxal, Tart, Mal, and Acet in leaves (F_{Exp}=4.34-8.35<F_{Crit}=3.78-4.32), Tart, Mal, and Fum in grapes (F_{Exp}=5.77-12.90<F_{Crit}=4.32-9.00), and in Oxal, Tart, and Mal in musts (F_{Exp}=4.42-35.11<F_{Crit}=3.78-4.32). In these cases, the results do not present significant differences (T_{Exp}=0.24-1.79<T_{Crit}=1.86-4.19) for 95 % confidence level. Therefore, it can be concluded that an external calibration is an acceptable tool for quantifying organic acids by HPLC-DAD in leaves, grapes, and musts of *Vitis vinifera* L.

For instance, graphical comparison of the external calibration curves and standard addition in a pooled leaf, must, and grape samples of *Vitis vinifera* L. are shown in *Fig. IV.1* to *Fig. IV.3* in **Annex IV**.

3.1.6.2.2.4 ANALYTICAL METHOD EVALUATION

External calibration curves in the different plant materials of *Vitis Vinifera* L. were done at different ranges of concentrations. Regression equations of these compounds are summarized in *Tables 3.6-3.8.* These equations were obtained facing analyte signal from chromatograms peak areas from photodiode array detector chromatograms. In these cases, correlation coefficients above 0.9995 were achieved for all studied analytes.

A second statistical analysis was necessary on the random distribution of the residues to verify if the linear regression models proposed for each compound are acceptable. This assumption must meet that if the data distribution is normal, the arithmetic means of the residues, $\bar{r}$, is expected to be close to 0. In addition, the standard deviation of the residues σ(r), was equal or less tan global standard deviation, σ_{TOT}, and average residue, $|\bar{r}|$, was equal to or lower than σ(r). *Tables 3.6-3.8* show the

statistical analysis results on the random distribution of the residues and verify the requirements to assert that the proposed equations of linear regression are adequate to obtain data.

Table 3.6. *Calibration curves and statistical analysis on the random distribution of the residues for quantifying of organic acids in leaf samples of* Vitis vinifera L.

Analytes	Calibration Range (mg L^{-1})	Slope ± error	Intercept ± error	Correlation coefficient (r)	σ_{TOT}	$\bar{r}$	σ(r)	$\lvert\bar{r}\rvert$
Oxal	2.07-2,072.56	2.26 ± 0.05	47.79 ± 43.12	0.9992	83.93	0.00	68.53	58.24
Tart	0.25-2,090.00	13.80 ± 0.12	148.07 ± 131.04	0.9998	235.17	0.00	198.76	169.50
Mal	0.25-2,072.00	6.81 ± 0.04	39.32 ± 35.55	0.9998	94.76	0.00	85.72	70.30
Acet	1.00-1,035.00	5.43 ± 0.03	12.09 ± 10.18	0.9999	25.35	0.00	22.35	16.99
Cit	0.50-282.00	14.23 ± 0.12	-30.21 ± 11.90	0.9998	30.46	0.00	26.86	24.24
Succ	0.50-25.22	3.52 ± 0.04	6.07 ± 0.44	0.9997	0.83	0.00	0.68	0.54
Fum	0.05-0.25	2,523.00 ± 237.03	19.31 ± 4.66	0.9997	7.57	0.00	5.87	4.49

Table 3.7. *Calibration curves and statistical analysis on the random distribution of the residues for quantifying of organic acids in must samples of* Vitis vinifera L.

Analytes	Calibration Range (mg L^{-1})	Slope ± error	Intercept ± error	Correlation coefficient (r)	σ_{TOT}	$\bar{r}$	σ(r)	$\lvert\bar{r}\rvert$
Oxal	0.05-1,065.00	22.41 ± 0.23	-19.48 ± 111.69	0.9997	208.17	0.00	169.97	125.56
Tart	0.25-2,090.00	14.17 ± 0.10	209.86± 111.37	0.9998	196.97	0.00	160.83	133.74
Mal	0.25-2,072.00	5.47 ± 0.05	50.05 ± 52.50	0.9997	105.81	0.00	89.43	78.86
Acet	1.00-258.75	4.09 ± 0.05	10.30 ± 6.93	0.9997	11.25	0.00	8.71	6.77
Cit	0.50-564.00	8.16 ± 0.07	-34.79 ± 18.94	0.9998	35.06	0.00	28.63	28.27
Succ	0.50-100.86	12.39 ± 0.14	-7.44 ± 6.70	0.9997	12.09	0.00	9.87	7.33
Fum	0.05-2.75	2,321.66 ± 29.91	-29.55 ± 44.79	0.9995	75.11	0.00	63.48	44.98

Table 3.8. *Calibration curves and statistical analysis on the random distribution of the residues for quantifying organic acids in grape samples of* Vitis vinifera *L.*

Analytes	Calibration Range (mg L^{-1})	Slope ± error	Intercept ± error	Correlation coefficient (r)	σ_{TOT}	$\bar{r}$	σ(r)	$\lvert\bar{r}\rvert$
Oxal	0.05-1,065.00	22.41 ± 0.23	-19.48 ± 111.69	0.9997	208.17	0.00	169.97	125.56
Tart	0.25-2,090.00	14.17 ± 0.10	209.86± 111.37	0.9998	196.97	0.00	160.83	133.75
Mal	0.25-1,491.00	5.59 ± 0.04	18.85 ± 28.71	0.9998	55.41	0.00	45.24	36.97
Acet	1.00-100.40	4.32 ± 0.07	1.67 ± 4.13	0.9997	5.30	0.00	3.74	3.15
Cit	0.50-304.60	7.44 ± 0.06	-0.07 ± 7.57	0.9998	14.61	0.00	11.93	8.62
Succ	0.50-25.22	11.34 ± 0.24	6.07 ± 0.44	0.9995	4.49	0.00	3.18	2.69
Fum	0.05-0.54	2,277.55 ± 25.68	0.72 ± 3.34	0.9998	11.25	0.00	7.96	6.79

The LOQs define the sensitivity of this HPLC-DAD method. There were no leaves, musts, or grapes target analytes-free, decreasing concentrations of calibration standards were included in a calibration curve. LOQs were set as the first level concentration level of these calibration curves. Based on this, LOQs were determined as 0.05 mg L^{-1} for Oxal, 0.25 mg L^{-1} for Tart, 0.25 mg L^{-1} for Mal, 1.00 mg L^{-1} for Acet, 0.50 mg L^{-1} for Cit, 0.50 mg L^{-1} for Succ, and 0.05 mg L^{-1} for Fum. The precision of the developed method was calculated for low, medium, and high standard calibration levels in the proposed calibration curves. The precision was also calculated in terms of repeatability and intermediate precision after analyzing five replicates ($n=5$) during five consecutive days; everyone calculated and expressed as RSD (%).

Table 3.9. *Summary of validation parameters of HPLC-DAD analytical methodology.*

	LOQ (mg L^{-1})	Repeteability (%RSD)			Intermediate Precision (%RSD)		
		Low Level	Medium Level	High Level	Low Level	Medium Level	High Level
Oxal	0.05	3.42	3.74	1.79	3.19	6.24	2.45
Tart	0.25	5.13	1.81	2.10	2.33	1.12	1.69
Mal	0.25	8.31	2.60	1.98	6.50	6.39	4.59
Acet	1.00	7.84	2.33	0.86	4.54	4.57	9.34
Cit	0.50	1.10	3.47	4.87	1.23	11.24	3.85
Succ	0.50	0.36	2.72	2.10	0.36	6.64	5.77
Fum	0.05	4.10	2.02	1.02	0.95	4.3	8.59

As observed in *Table 3.9*, the repeatability of measurements was between 0.36-8.31% and the intermediate precision among 0.36-11.24% for all the analytes. Therefore, it can be concluded that the method precision was within the acceptable range in this study.

Finally, the efficiency of sample preparation is usually evaluated using recovery assessment in two concentration levels, analyzing five replicates (*n=5*) per level as described in section 3.8.2.1.2. Considering the results observed in *Table 3.10*, the organic acid extraction procedure in *Vitis vinifera* L. leaves provides recoveries between 78.91-96.55% and 79.17-101.53% for low and high levels, respectively.

Table 3.10. *Recovery (%) and standard deviation of organic acids in two concentrations levels in a pooled leaf of* Vitis vinifera *L.*

Recovery (%)	Low Level	High Level
Oxal	79.26 ± 3.17	79.17 ± 2.85
Tart	90.89 ± 5.61	98.07 ± 4.17
Mal	91.40 ± 3.75	99.79 ± 3.05
Acet	95.04 ± 5.84	100.37 ± 3.85
Cit	96.55 ± 5.11	95.66 ± 6.94
Succ	90.21 ± 4.94	101.53 ± 3.85
Fum	78.91 ± 3.23	98.89 ± 4.23

Likewise, *Table 3.11* shows that the recoveries obtained in the organic acid extraction procedure in *Vitis vinifera* L. grape samples were 91.77-103.41% and 93.18-105.75% for low and high levels, respectively. Consequently, it can be concluded that the developed organic acid extraction procedure in grape and leaf samples of *Vitis vinifera* L. provides a good recovery.

Table 3.11. *Recovery (%) and standard deviation of organic acids in two concentrations levels in a pooled grape of* Vitis vinifera *L.*

Recovery (%)	Low Level	High Level
Oxal	91.77 ± 8.16	101.80 ± 4.22
Tart	98.92 ± 9.40	101.75 ± 4.42
Mal	99.65 ± 7.29	102.12 ± 4.43
Acet	103.41 ± 3.62	100.77 ± 3.11
Cit	95.20 ± 5.92	99.64 ± 4.65
Succ	98.89 ± 4.79	105.75 ± 1.00
Fum	100.26± 1.58	93.18 ± 6.33

3.1.6.3 FREE AMINO ACIDS QUANTIFICATION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO FLUORESCENCE DETECTOR (HPLC-FLD)

3.1.6.3.1 MATERIALS

3.1.6.3.1.1 STANDARD AND REAGENTS

Different manufacturers supplied free AAs standards; L-aspartic (Asp), L-glutamic (Glu), L-glutamine (Gln), L-(+)-arginine (Arg), and L-alanine (Ala) from TCI (Tokyo, Japan), and L-(+)-asparagine (Asn), ɣ-aminobutyric acid (GABA) from Alfa Aesar (Karlsruhe, Germany), and β-aminobutyric acid (BABA), and α-aminobutyric acid (AABA) from Sigma-Aldrich (Steinheim, Germany). *DL*-2-aminoadipic acid from Fluka Analytical (Charlotte, NC, USA) was used as an internal standard (IS).

Organic solvents used for the mobile phase, sample extraction, and injection diluent were spectrally pure for LC-MS. Acetonitrile (ACN), methanol (MeOH) and ethanol (EtOH) from Scharlau (Sentmenat, Spain), sodium monophosphate (Na_2HPO_4) and 85% phosphoric acid (H_3PO_4) from Merck (Darmstadt, Germany), sodium azide (NaN_3) from Sigma-Aldrich (Madrid, Spain), sodium tetra-borate decahydrate ($Na_2B_4O_7.10H_2O$) from Panreac (Barcelona, Spain) were used. Hydrochloric acid (HCl, 37%) from Fisher Scientific (Ghent, Belgium) stabilized standard solutions.

Ultra-high purity water was obtained from tap water pre-treated by Elix20 reverse osmosis and purified by a Milli-Q filtration system from Millipore (Bedford, USA). Moreover, the mobile phase was filtered through 0.22 µm Polytetrafluoroethylene (PTFE) filters from Millipore (Watford, Ireland).

3-mercaptopropionic acid in borate buffer (OPA) and 9- fluorenylmethyl chloroformate (FMOC) in acetonitrile, and borate buffer solution obtained from Agilent Technologies (Santa Clara, CA, USA) were used for free amino acids derivatization. These reagents were replaced weekly due to the observed degradation suffered at room temperature in the HPLC-FLD equipment during the measurements.

3.1.6.3.1.2 PREPARATION OF STANDARD SOLUTIONS

For AAs quantification by HPLC-FLD, standard stock solutions for each amino acid containing around 10,000 mg L^{-1} and 1,000 mg L^{-1} were prepared in 0.1 N of HCl, and from these solutions, individual standard dilutions of 1 mg L^{-1} and 5 mg L^{-1} were prepared. All standard solutions were stored frozen at -20°C.

Mixed solutions containing 0.005 mg L^{-1}, 0.01 mg L^{-1}, 0.05 mg L^{-1}, and 0.1 mg L^{-1} were prepared in the mobile phase at initial conditions to determine the LOQs.

The quantification of amino acids was performed using calibration standards solutions contained between 0.2-1,500 mg L^{-1} were prepared in EtOH. To analyze the real samples, the standard calibration solutions were diluted at 1:10, 1:20, and 1:50 for leaves, must, and grape, respectively. Depending on the range of each amino acid in the studied matrices, the range of calibration curves was adjusted between 0.005-10 mg L^{-1} for leaves, 0.005-100 mg L^{-1} for musts, and 0.005-10 mg L^{-1} for grape paste. All measurements on calibration curves were done in triplicate.

For determining the endogenous free amino acids in the different plant materials of *Vitis vinifera* L., a previous standard addition calibration method was made to quantify a pooled leaf, must, and grape beforehand. Spiking pooled leaf or must/grape paste from T0A sampling of control (C) treatments were used. The pools with leaves were spiked with increasing volumes of standards prepared at 129.20 mg L^{-1} of Asp, 200.75 mg L^{-1} of Glu, 50.23 mg L^{-1} of Asn, 306.28 mg L^{-1} of Gln, 55.97 mg L^{-1} of Arg, 202.00 mg L^{-1} of Ala, 203.25 mg L^{-1} of GABA, 23.06 mg L^{-1} of BABA. Similarly, the

pooled must was spiked with a solution containing 1,590 mg L^{-1} of Asp, 3,550 mg L^{-1} of Glu, 580 mg L^{-1} of Asn, 5,990 mg L^{-1} of Gln, 47,961 mg L^{-1} of Arg, 2,300 mg L^{-1} of Ala, 2,370 mg L^{-1} of GABA and 2,040 mg L^{-1} of BABA. The pooled grape was spiked with a mixed standard solution containing 880 mg L^{-1} of Asp, 1,330 mg L^{-1} og Glu, 450 mg L^{-1} of Asn, 2,030 mg L^{-1} of Gln, 2,710 mg L^{-1} of Arg, 1,050 mg L^{-1} of Ala, 450 mg L^{-1} of GABA, 84,864 mg L^{-1} of BABA. In both studies, the internal standard contains 1,360 mg L^{-1}.

3.1.6.3.2 METHODS

3.1.6.3.2.1 EXTRACTION METHOD PROCEDURE

One-hundred leaf samples, forty must and forty grape samples of *Vitis vinifera* L. (cv. Tempranillo) previously stored at -40°C were analyzed. In addition, the three commercial biostimulants (ST, GT, and BF) were characterized by the analytical methodology developed HPLC-FLD. All measurements were made in triplicate.

- **LEAVES**

Free amino acids were extracted according to the method described by De Diego *et al*. (2013)[361] with some minor modifications. Plant material collected at T0A, T24A, T0B, T24B, and TF described in section 3.3.1, were pooled, homogenized, and lyophilized. Aliquots with 100 mg of each sample and 5 µL of the internal standard were placed in a 2 mL vial and dropped with 1 mL of EtOH. After shaking the extracts, they were centrifuged at 10,000 rpm for 10 min at 4ºC.

Samples were re-extracted for 10 min in an additional 1 mL of EtOH. At initial conditions, supernatants were collected and diluted at 1:10 in the mobile phase. Finally, samples were filtered through 0.22 µm of PVDF syringe filters.

- **MUST AND GRAPES**

The determination of free amino acids in grapes and musts was performed by direct injection in an HPLC-FLD system as previously described by Kelly *et al*. (2010)[362] with some minor changes.

The AAs analysis in berries was carried out by adding 980 µL of EtOH and 20 µL of internal standard stock solution to 200 mg of sample. The mixtures were stirred for 10 min to improve the total extraction of amino acids. Then, samples were centrifuged at 10,000 rpm at 4ºC for 10 min, and after that, diluted at 1:50 with the mobile phase.

In musts, 10 µL of internal standard stock solution were added to 2 mL of must and then diluted at 1:20 in the mobile phase.

Before injection in the HPLC-FLD system, all samples were filtered through 0.22 µm of PVDF syringe filters.

- **BIOSTIMULANTS**

Both ST and GT biostimulants were diluted at 1:2 with EtOH and 1:5 ratio for BF. Before the injections, all samples were filtered through 0.22 µm of PVDF syringe filters.

3.1.6.3.2.2 INSTRUMENTAL ANALYSIS

The analytical method based on the HPLC-FLD was carried out on an Agilent 1100 HPLC system with a 1260 series Agilent quaternary pump and 1260 series automatic liquid sampler from Agilent Technologies (Santa Clara, USA). The system was coupled to an Agilent 1260 fluorescence detector (FLD) that was operated at an excitation wavelength of 230 nm and an emission wavelength of 460 nm. The analysis was performed by injecting 20 µL of a derivatized sample using an injection program previously described by Agilent Technologies[363,364]. Free amino acids derivatization occurred in the loop of the injection port using an injection program. The injection diluent was 100 mL mobile phase A, prepared with Na_2HPO_4 (10 mM), $Na_2B_4O_7{\cdot}10H_2O$ (10 mM) and NaN_3 (5 mM), plus 0.4 mL concentrated H_3PO_4. This solution was kept in refrigeration at 4°C and replaced weekly.

Chromatographic separation was carried out using an InfinityLab Poroshell 120 HPH-C18 column (2.7 µm, 100 × 4.6 mm i.d.) protected with an InfinityLab Poroshell 120 guard column (2.7 µm, 5 × 4.6 mm i.d) both from Agilent Technologies (Santa Clara, CA, USA). The column temperature was set at 40°C, and a flow rate of 1 mL min^{-1} was used during the analysis.

The mobile phase was composed in A [Na_2HPO_4 (10 mM), $Na_2B_4O_7.10H_2O$ (10 mM, pH 8.2) and NaN_3 (5 mM)], and B contained ACN/MeOH/H_2O (45:45:10 %v/v/v). The elution consisted of a 20 min of mobile phase gradient; 2% (B) for 0.35 min, 2-57% (B) for 13.05 min, 57-100% (B) for 0.10 min, 100% (B) for 2.20 min; 100-2% (B) for 0.10 min; and 2% (B) for 2.20 min. The column was equilibrated with initial composition of the mobile phase for 9 min before each analysis.

Agilent LC ChemStation software (Agilent Technologies, Inc., Palo Alto, CA, USA) was used for data analysis. Known concentration standards of each component were also examined under the same conditions. The calibration curves were obtained normalizing the signal from the analyte with the internal standard and facing this ratio from areas of FLD chromatographic peaks.

Before free amino acids extraction, plant material of *Vitis vinifera* L. was lyophilized using a LyoQuest-85/230V 50 Hz Lyophilizer from Telstar (Tokyo, Japan) and crushed in a Mikro-dismenbrator (B. Braun Biotech, Melsungen, Germany). Graduated Eppendorf™ polypropylene microtubes from Fischer Scientific (Ghent, Belgium) were used to process samples and extract the free amino acids in the matrices. Samples and standards were centrifuged in a 5415R Eppendorf™ microcentrifuge from Marshall Scientific (Hampton, USA) and filtered through 0.22 µm PVDF filters.

3.1.6.3.2.3 MATRIX EFFECT EVALUATION

As well as the analytical methodologies detailed above, there is no analyte-free vegetal material from *Vitis vinifera* L. to quantify free amino acids by adding analytical standards to a blank matrix at known quantities. For this reason, the ME of the different plant materials of *Vitis vinifera* L. was evaluated as previously discussed.

Each concentration level in the standard addition and the external calibration methods were carried out in triplicate ($n=3$), and relative standard deviations obtained were less than 5% for all analytes studied. Statistical comparison at 95% of a confidence level of the matrix effect of preparing analytical standards in EtOH and the standard addition method were performed.

As shown in **Annex V** (*Tables V.1-V.3)*, the ME is within ±5% for all the analytes studied. The F-test study homogeneous variances for Asp, Arg, and BABA in leaves (F_{Exp}=1.03-5.50<F_{Crit}=9.55),

Asp, Glu, Arg, GABA, and BABA in grape paste (F_{Exp}=1.31-2.78<F_{Crit}=6.94-19.25) and Asp, Glu, Asn, Gln, Arg, Ala, GABA and BABA in musts (F_{Exp}=1.22-5.09<F_{Crit}=5.14-19.25) has been verified. For these instances, the results obtained by the Student t-test are statistically comparable (T_{Exp}=0.02-2.137<T_{Crit}=1.90-2.45) at 95% confidence level for all the analytes in each matrix of *Vitis Vinifera* L. Consequently, non-homogeneous variances have been observed in leaves for Glu, Asn, Gln GABA and Ala (F_{Exp}=6.70-22.729<F_{Crit}=5.79-9.55), and Asn, Gln, and Ala in grapes (F_{Exp}=9.54-42.74<F_{Crit}=6.94). In these cases, the results do not present significant differences (T_{Exp}=0.01-1.86<T_{Crit}=4.10-4.27) for a 95% confidence level.

The values of the ME are within ±5% for all the analytes studied, so it can be concluded that an external calibration is an acceptable tool for quantifying these nine amino acids in leaves, grapes, and musts samples from *Vitis vinifera* L. using the proposed analytical methodology.

In addition, graphical comparison of both quantification methods in a pooled leaf, must, and grape samples of *Vitis vinifera* L. are shown in **Annex V** (*Fig. V.1-V.3)*.

3.1.6.3.2.4 ANALYTICAL METHOD EVALUATION

Consequently, external calibration curves in the studied plant material of *Vitis Vinifera* L. were built at different ranges and solvents as described in section 3.8.3.1.2. Regression equations of these compounds are summarized in T*ables 3.12-3.14. C*orrelation coefficients above 0.9987 were achieved for all analytes. In order to verify if the linear regression models proposed for each compound are acceptable, a second statistical analysis was necessary on the random distribution of the residues.

This assumption must meet that if the data distribution is normal, the arithmetic means of the residues, $\bar{r}$, is expected to be close to 0. In addition, the standard deviation of the residues σ(r) was equal or less tan global standard deviation, σ_{TOT}, and average residue, $|\bar{r}|$, was equal to or lower than σ(r). *Tables 3.12-3.14* show the statistical analysis results on the random distribution of the residues and verify the requirements to assert that the proposed equations of linear regression are adequate to obtain data.

Table 3.12. *Calibration curves and statistical analysis on the random distribution of the residues for quantifying amino acids in leaf samples of* Vitis vinifera *L.*

Analytes	Calibration Range (mg L^{-1})	Slope ± error	Intercept ± error	Correlation coefficient (r)	σ_{TOT}	$\bar{r}$	σ(r)	$\lvert\bar{r}\rvert$
Asp	0.02-1.05	6.20 ± 0.10	0.28 ± 0.05	0.9995	0.09	0.00	0.07	0.06
Glu	0.02-1.05	7.51 ± 0.10	0.01 ± 0.06	0.9995	0.10	0.00	0.09	0.07
Gln	0.01-7.57	3.84 ± 0.06	0.32 ± 0.22	0.9996	0.38	0.00	0.30	0.24
Arg	0.01-0.51	10.77 ± 0.25	0.59 ± 0.07	0.9991	0.10	0.00	0.08	0.07
Ala	0.01-2.00	11.43 ± 0.13	1.05 ± 0.12	0.9997	0.21	0.00	0.17	0.16
GABA	0.01-5.30	15.31 ± 0.21	-0.84 ± 0.50	0.9996	0.94	0.00	0.77	0.63
BABA	0.01-0.51	13.38 ± 0.16	0.08 ± 0.04	0.9997	0.07	0.00	0.05	0.04

Table 3.13. *Calibration curves and statistical analysis on the random distribution of the residues for quantifying free amino acids in must samples of* Vitis vinifera *L.*

Analytes	Calibration Range (mg L^{-1})	Slope ± error	Intercept ± error	Correlation coefficient (r)	σ_{TOT}	$\bar{r}$	σ(r)	$\lvert\bar{r}\rvert$
Asp	0.01-2.00	5.64 ± 0.14	1.42 ± 0.13	0.9988	0.23	0.00	0.19	0.15
Glu	0.01-5.08	6.28 ± 0.05	0.79 ± 0.11	0.9998	0.23	0.00	0.20	0.16
Asn	0.01-2.03	7.41 ± 0.09	0.56 ± 0.08	0.9997	0.15	0.00	0.13	0.12
Gln	0.01-60.20	8.39 ± 0.11	4.64 ± 3.40	0.9996	6.04	0.00	5.11	4.36
Arg	0.01-74.41	12.26 ± 0.25	-5.48 ± 11.43	0.9992	16.61	0.00	7.59	7.46
Ala	0.01-14.89	16.09 ± 0.28	-0.78 ± 1.81	0.9991	4.11	0.00	5.29	3.06
GABA	0.01-14.94	12.1 ± 0.05	0.05 ± 0.31	0.9999	0.70	0.00	0.61	0.50
BABA	0.01-0.50	8.93 ± 0.11	0.27 ± 0.03	0.9997	0.05	0.00	0.04	0.03

Table 3.14. *Calibration curves and statistical analysis on the random distribution of the residues for quantifying free amino acids in grape samples of* Vitis vinifera *L.*

Analytes	Calibration Range (mg L^{-1})	Slope ± error	Intercept ± error	Correlation coefficient (r)	σ_{TOT}	$\bar{r}$	σ(r)	$\lvert\bar{r}\rvert$
Asp	0.01-4.00	1.96 ± 0.02	0.76 ± 0.04	0.9996	0.08	0.00	0.07	0.06
Glu	0.01-2.02	3.08 ± 0.05	0.68 ± 0.05	0.9995	0.08	0.00	0.07	0.05
Asn	0.01-1.02	4.47 ± 0.10	0.41 ± 0.04	0.9991	0.08	0.00	0.07	0.05
Gln	0.01-5.05	4.94 ± 0.05	0.22 ± 0.11	0.9996	0.34	0.00	0.31	0.27
Arg	0.01-4.05	8.70 ± 0.11	3.10 ± 0.19	0.9996	0.39	0.00	0.15	0.26
Ala	0.01-2.01	9.78 ± 0.19	1.35 ± 0.17	0.9990	0.35	0.00	0.29	0.25
GABA	0.01-4.04	12.83 ± 0.19	-0.06 ± 0.34	0.9994	0.69	0.00	0.23	0.50
BABA	0.01-0.50	3.5 ± 0.1	0.33 ± 0.03	0.9987	0.04	0.00	0.04	0.02

Although different solvents were used to build the calibration curves, the limits of quantification were similar. Therefore, LOQs for leaf calibration curves were determined as 0.01 mg L^{-1} for Asp, 0.01 mg L^{-1} for Glu, 0.01 mg L^{-1} for Asn, 0.01 mg L^{-1} for Gln, 0.01 mg L^{-1} for Arg, 0.01 mg L^{-1} for Ala, 0.01 mg L^{-1} for GABA and 0.01 mg L^{-1} for BABA.

The precision of the developed method was calculated for low, medium, and high standard calibration levels in the proposed calibration curves. The precision was also calculated in terms of repeatability and intermediate precision after analyzing five replicates (*n=5*) during five consecutive days, everyone calculated and expressed as relative standard deviation (%).

As observed in *Table 3.15*, the repeatability of measurements was between 0.57-8.80%, and the intermediate precision among 0.41-8.80% for all the analytes. Therefore, it can be concluded that the method precision was within the acceptable range in this study.

Table 3.15. *Summary of validation parameters of HPLC-FLD analytical methodology.*

	LOQ (mg L^{-1})	Repeteability (%RSD)			Intermediate Precision (%RSD)		
		Low level	Medium Level	High Level	Low level	Medium Level	High Level
Asp	0.01	6.54	1.47	4.06	0.73	4.51	6.44
Glu	0.01	5.25	7.30	7.67	2.68	7.64	2.52
Asn	0.01	1.16	6.06	2.67	6.89	7.04	6.62
Gln	0.01	6.16	6.60	0.57	1.19	3.33	0.41
Arg	0.01	0.86	1.37	4.94	0.60	8.42	1.67
Ala	0.01	4.29	8.19	7.35	0.92	6.97	2.61
GABA	0.01	1.65	4.37	8.40	0.97	4.94	2.74
BABA	0.01	8.80	8.01	4.79	8.65	7.34	8.80

Finally, the efficiency of the free amino acid extraction procedure in leaves and grapes of *Vitis vinifera* L. was also evaluated in terms of recovery assessment analyzing five replicates (n=5) per level.

As mentioned above, QC samples are quantified beforehand by standard addition and used to study the recovery of the proposed analytical methodology. Considering the results observed in *Table 3.16*, the free AAs extraction procedure in leaves of *Vitis vinifera* L. provides recoveries between 82.90-102.93% and 84.33-103.05% for low and high levels, respectively.

Likewise, *Table 3.17* shows the recoveries obtained in the free amino acid extraction procedure in the grape paste of *Vitis vinifera* L. In these cases, the recoveries ranged between 86.01-99.20% and 95.29-99.75%, respectively. Consequently, it can be concluded that the developed free AAs extraction procedure in leaves and grape samples of *Vitis vinifera* L. provides an acceptable recovery.

Table 3.16. *Recovery (%) and standard deviation of amino acids in a pooled leaf of* Vitis vinifera *L.*

Recovery (%)	Low level	High level
Asp	82.90 ± 3.57	84.71 ± 0.51
Glu	96.46 ± 0.30	84.33 ± 0.34
Asn	84.02 ± 0.52	93.84 ± 0.54
Gln	95.64 ± 2.62	93.01 ± 3.70
Arg	90.22 ± 2.04	87.23 ± 1.51
Ala	96.58 ± 4.47	86.28 ± 0.36
GABA	97.59 ± 3.15	103.05 ± 1.41
BABA	102.93 ± 0.55	95.81 ± 2.90

Table 3.17. *Recovery (%) of amino acids in a pooled grape of* Vitis vinifera *L.*

Recovery (%)	Low level	High level
Asp	95.89 ± 7.24	99.11 ± 8.42
Glu	95.12 ± 5.50	99.75 ± 5.88
Asn	86.10 ± 5.08	98.87 ± 6.32
Gln	86.01 ± 6.94	95.29 ± 8.19
Arg	99.20 ± 5.70	98.38 ± 5.16
Ala	91.49 ± 3.56	98.66 ± 1.83
GABA	93.00 ± 5.23	97.40 ± 2.92
BABA	95.44± 4.89	98.03 ± 7.38

3.2 EXPERIMENTAL CONDITIONS FOR *ARABIDOPSIS THALIANA*

3.2.1 PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis thaliana (accessions Col-0) was used as plant material to study the mechanism and mode of action of the three commercial biostimulants used in the vineyard experiment (Section 3.1.2). Arabidopsis seeds were surface- sterilized with a solution of 70% of EtOH for 10 min, then washed three times with sterilized water, and immediately after that with a 4% sodium hypochlorite for 4 min and additional four washes with sterilized water[365]. The sterile seeds were transferred to a Petri dish (92 x 16 mm) containing sucrose-free half-strength solid Murashige and Skoog medium containing 0.8% Phytotechlab M519. The plates were kept in darkness for four days at 4ºC. Then, the sterile seeds were placed under light conditions into the growth chamber for seven days at a relative humidity of 60% and a photoperiod of 16/8 h of light/dark at 22ºC and 120 µmol s^{-1} m^{-2} of light intensity[366].

Eleven days after stratification (DAS), one hundred sixty seedlings of similar size were transferred into plastic pots (70 mm x 70 mm x 65 mm, Poppelman, TEKU, DE) containing 130 g of freshly sieved soil (Substrate 2; Klasmann-Deilmann GmbH, Geeste, Germany) and watered to full soil-water holding capacity (FC). The substrate water content (%) was calculated as described by Marchetti *et al.* (2019)[365]. After four days of acclimation from the transfer, the seedlings were divided into two groups; one well-watered in which the plants were kept at 80% FC, and another group with progressive drought conditions (PD) obtained by removing the water supply, which ended with 50% field capacity (PD) at the end of the experiment. The pots were weighed daily to maintain the substrate moisture regime, and the necessary water was replenished based on water consumption per pot.

For studying the mechanism of action of the three commercial bisostimulants (ST, GT, and BF) applied in *Vitis vinifera* and described in section 3.1.1.1, the one hundred sixty Arabidopsis seedlings transferred to the plastic pots were located in standard blue trays with 20 plants each. A total of 8 trays were used, half for the well-watered plants and another half for the drought treatment. Each group was divided into four subgroups in which the plants were sprayed with ST,

GT, or BF or with distiller water (C) as a negative control. Two foliar applications were performed during phenotyping on days six and thirteen with a solution of 3% (v/v) with 0.1% tween 20.

3.2.2 PHENOTYPING PLATFORM, EXPERIMENTAL SETUP AND ASSAY CONDITIONS

All plants were grown in a climate-controlled growth chamber with cool-white and far-red LED lighting into the OloPhen Platform using the PlantScreen™ Compact system (Photon Systems Instruments, Brno, Czech Republic). Growth conditions were established to simulate a short-day regime with 22ºC /20ºC in a 12h/12h light/dark cycle, a relative humidity (RH) of 55%, and 130 µmol m^{-2} s^{-1} of light intensity[365,366]. The PlantScreen™ Compact system consists of a conveyor belt system equipped with different sensors for non-invasive phenotyping, including top-view red-blue-green (RGB), infrared (IR) and chlorophyll fluorescence (ChlF) cameras positioned at 50 cm height from the plants.

All plants were automatically phenotyped for RGB and kinetic ChlF traits for 17 days (*Fig. 3.6*), from 26 to 42 DAS. Imaging information for each tray was performed once per day every two days. Trays were transported within the PlantScreen™ Compact system through conveyor belts between the light-isolated imaging cabinets, weighing and watering station, and the dark/light acclimation chamber. Two independent rounds were performed. The first round consisted of a fast measurement performed in the middle of the day for weighing and watering and, immediately after the image analysis using the IR followed by RGB top-view camera. The second round consisted of an initial 15 min dark-adaptation period inside the acclimation chamber followed by ChlF imaging. The system automatically captured and stored the images in a database server. The images data of the whole experiment were automatically stored in the PlantScreen™ database and exported using the PlantScreen Data Analyzer software by PSI (Photon Systems Instruments, Brno, Czech Republic).

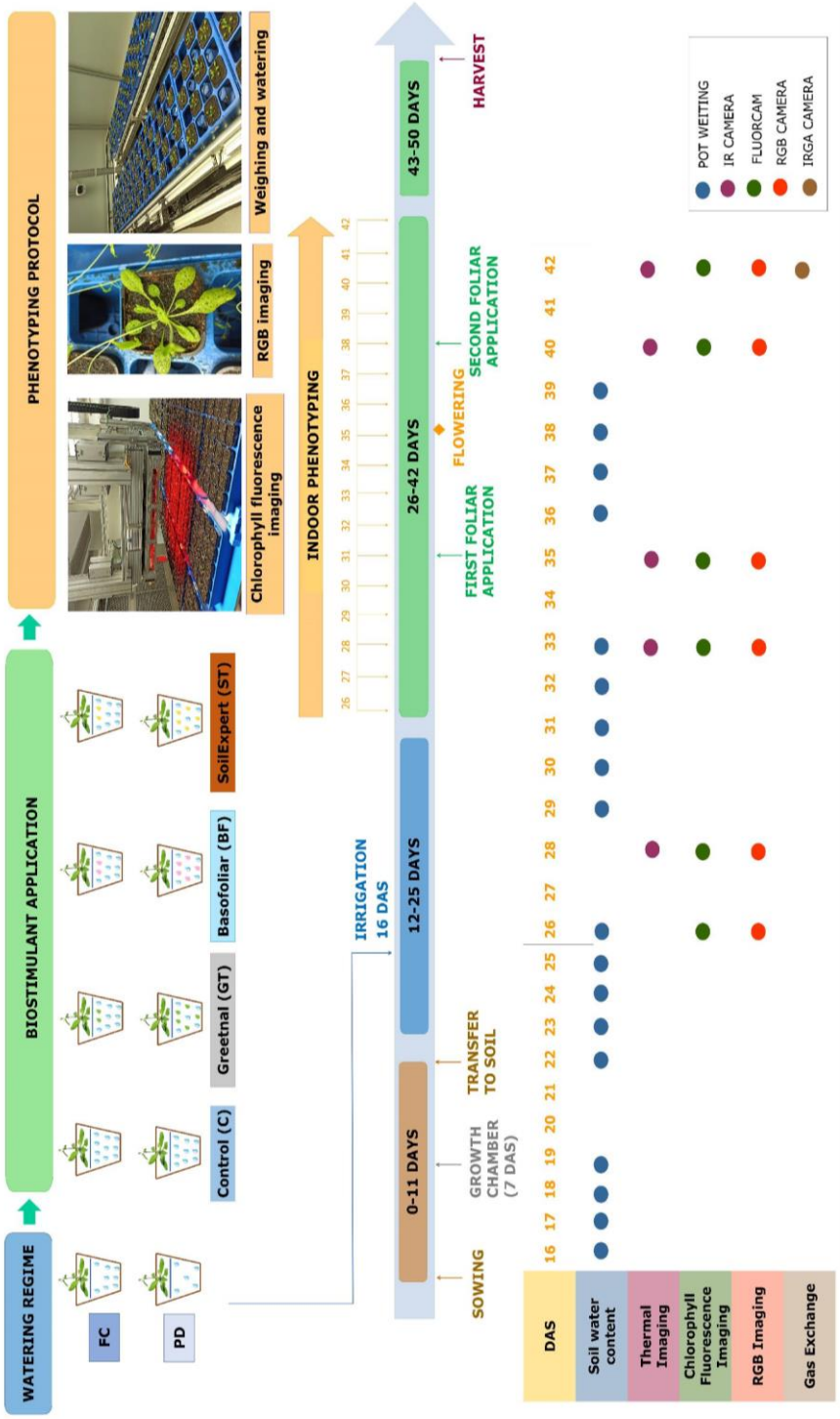


Figure 3.6. *Experimental design for the foliar application of biostimulants in* Arabidopsis thaliana *and growth and test conditions within the PlantScreen™ Compact system.*

3.2.3 SAMPLING AND SAMPLE STORAGE

During the drought period, two samplings were carried out; 24 h after the first foliar application (32 DAS) and 24 h after the second foliar application (39 DAS), in which three seedlings per variant were for the metabolite quantification and additional four seedlings to determine the water status. The remaining six plants were kept until the end of the plant life cycle to evaluate the plant yield.

All the materials used for the collections were previously sterilized with a solution of 70% EtOH to avoid contamination. Plant materials were cut and placed in 5 mL eppendorfs, frozen using liquid nitrogen, and immediately stored at -80ºC. Finally, the plant material was lyophilized using Martin Christ Beta 1-8 LDPlus lyophilizer (Sigma-Aldrich, Steinheim, Germany).

3.2.4 WATER STATUS DETERMINATION

Relative water content (RWC, %) was determined in three Arabidopsis seedlings as biological replicates the first day of the phenotyping protocol (day 26 DAS), at 27 DAS, 32 DAS and 39 DAS using *Eq. 3.8*[367]:

$$\mathrm{RWC}\ (\%) = \frac{(\mathrm{FW} - \mathrm{DW})}{(\mathrm{TW} - \mathrm{DW})} \cdot 100 \qquad \textit{(Eq 3.8)}$$

Where FW is the fresh weight of the leaf, TW is the turgid mass of the leaf estimated after 24 h of imbibition in water, and DW is the dry weight of the leaf after 48 h at 60ºC.

3.2.5 MORPHOLOGICAL PARAMETERS

Morphological parameters, such as rosette area (mm), perimeter (mm), compactness, isotopy, and eccentricity, were monitored from 26 to 42 DAS seedlings using a top-view RGB camera. The roundness was calculated as the ratio between the area and the perimeter of the plant surface. This index explained general information about plant shape. Rotational Mass Symmetry (RMS) was also used as an alternative approach for evaluating plant shape. Computation is based on fitting ellipses with the same second central moments as the area covered by an actual plant. RMS was calculated as the ratio between the focus of the ellipses and its major axis length without considering the

distribution of leaf mass. Finally, Slenderness of Leaves (SOL) was calculated as the ratio between square leaf lengths, as a sum of distances from plant center to the end of leaf blades and plant area.

3.2.6 GAS EXCHANGE PARAMETERS

The instant foliar transpiration rate E (mmol H_2O m^{-2} s^{-1}), the stomal conductance to water vapor g_s (mmol H_2O m^{-2} s^{-1}), the instantaneous net photosynthesis assimilation rate A (µmol CO_2 m^{-2} s^{-1}), and the internal foliar CO_2 concentration in the substomatic cavities C_i (ppm CO_2) were measured in fully expanded Arabidopsis leaves at 42 DAS. They were located into the photosynthesis chamber connected to a portable open-flow gas exchange system (CIRAS 3, PP Systems Inc., Amesbury, MA). All measurements were performed under artificial PAR (400 µmol photons m^{-2} s^{-1}) with red, green, and blue lights set at 38, 37, and 25% of PAR, respectively. The CO_2 supplying was performed with CO_2 cartridges (8 g) set to 400 µmol CO_2 mol^{-1} air, whereas the cuvette temperature oscillated between 23 to 25 °C, and the vapor pressure deficit was maintained at approximately 1.0 kPa. The measurements were performed in five plants per variant as biological replicates.

Likewise, the instantaneous water efficiency (WUE) was determined as the relation between instantaneous net photosynthesis and instant foliar transpiration to determine stomata's effectiveness in enhancing or maximizing photosynthesis by reducing water loss through transpiration.

In addition, maximum leaf temperature (Tmax., ºC), minimum leaf temperature (Tmin., ºC), and average foliar leaf temperature (Ftemp., ºC) were determined using an IR camera from 26 to 42 DAS as an additional indirect measurement of the plant transpiration.

3.2.7 CHLOROPHYLL FLUORESCENCE IMAGING

Chlorophyll fluorescence (ChlF) measurements were performed using FluorCam FC-800MF pulse amplitude modulated (PAM) chlorophyll fluorometer (Photon Systems Instruments, Drásov, Czech Republic) at room temperature at 26, 28, 33, 35, 40, and 42 DAS.

The ChlF imaging station is mounted in a module with an LED light panel and a high-speed charge-coupled device camera (pixel resolution of 720×560, frame rate 50 fps and 12-bit depth) positioned

in the middle of the light panel. The LED panel was equipped with 3×64 orange-red (618 nm) and 64 cool-white LEDs (6,500 K), distributed equally over 75×75 cm. Modulated light of known wavelength was applied to detect the ChlF signal. Three types of light sources were used: (1) PAM short-duration measuring flashes (33 µs) at 618 nm, (2) orange-red (618 nm) and cool-white (6,500 K) actinic lights with maximum irradiance 440 µmol m^{-2} s^{-1} and (3) saturating cool-white light with maximum irradiance 3,000 µmol m^{-2} s^{-1}. Plant trays were automatically loaded into the light-isolated imaging cabinet of PlantScreen™ Compact system with a top-mounted LED light panel.

The ChlF protocol is described by Awlia *et al.* (2016)[368] with minor modifications. After the 15 min dark-adaptation period, when PSII reaction centers opened, the trays were automatically transported to the ChlF imaging cabinet. A 5 s flash of light with an intensity of 0.5 µmol m^{-2} s^{-1} was applied to measure the minimum level of fluorescence image (Fo), followed by a saturation pulse of 0.8 s (with an irradiance of 6,000 µmol m^{-2} s^{-1}) were used to determine the maximum fluorescence image (Fm). Plants were relaxed in the dark for 3 s and then subjected to 70 s of cool-white actinic lights to drive photosynthesis and measure the peak rise in fluorescence (Fp). These conditions were used in both ChlF imaging techniques using quenching kinetics and light curve protocol.

For quenching kinetics protocol, additional saturation pulses were applied at 8, 18, 28, 48, 68 s during actinic illumination for 3 min, corresponding to L1, L2, L3, L4, and Lss states at a constant photon irradiance of 500 µmol m^{-2} s^{-1} to obtain the light-adapted initial fluorescence (Fo') and steady state-fluorescence yield (Fs). Then a saturating blue light pulse (6,000 µmol m^{-2} s^{-1}, 0.8 s) was applied to acquire the maximum fluorescence in the light-adapted state (Fm'), and the level of ChlF was measured just before the saturation pulse was considered the steady-state fluorescence in the light-adapted state (Ft).

Finally, the software performed the calculation of maximum photosystem II (PSII) quantum yield (QY_max), steady-state PSII quantum yield (QY_Lss), steady-state non-photochemical quenching (NPQ_Lss), coefficient of photochemical quenching in the steady-state estimate of the fraction of open PSII reaction centers PSIIopen/(PSIIopen+ PSIIclosed) (*q*P_Lss), coefficient of non-photochemical quenching in steady-state (*q*N_Lss), and fluorescence decline ratio in steady-state (Rfd_Lss) as described in *Table VI.1* of **Annex VI**[369].

3.2.8 BIOMETRIC AND GROWTH PARAMETERS

At 32 and 39 DAS, four plants per variant were collected as biological replicates, and the aerial biomass was determined using *Eq. 3.9.* Shoots were cut 1 cm above ground level in three or four plants per replicate.

$$\text{Biomass (\%)} = \frac{DW}{FW} * 100 \qquad \textit{(Eq 3.9)}$$

Where, DW is the dry weight and FW is the fresh weight of the Arabidopsis rosette expressed in mg.

3.2.9 *ARABIDOPSIS THALIANA* PRODUCTION PARAMETERS AND SEED OIL QUALITY

3.2.9.1 YIELD PARAMETERS

Fifty days after the plant's sowing at the total senescence phase, the fruits from six plants per variant were harvested. The seeds were separated from the silique to determine the total number (Tot_number) of seeds, the total weight seeds (Tot_weight, mg), and the weight (mg) of 1,000 seeds (1,000_seeds) as variables associated with yield.

3.2.9.2 DETERMINATION OF FATTY ACIDS METHYL ESTERS (FAMEs) BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

3.2.9.2.1 MATERIALS

3.2.9.2.1.1 STANDARDS AND REAGENTS

A reference material (CRM) certified in accordance with ISO 17034 and ISO 17025 obtained from Merck (Darmstadt, Germany) was used as standard. The mixture consisted of methyl butyrate (400 µg mL^{-1}), methyl hexanoate (400 µg mL^{-1}), methyl octanoate (400 µg mL^{-1}), methyl decanoate (400 µg mL^{-1}), methyl undecanoate (200 µg mL^{-1}), methyl laurate (400 µg mL^{-1}), methyl tridecanoate

(200 µg mL^{-1}), methyl myristate (200 µg mL^{-1}), methyl pentadecanoate (200 µg mL^{-1}), methyl cis.10-pentadecanoate (200 µg mL^{-1}), methyl palmitate (600 µg mL^{-1}), methyl palmitoleate (200 µg mL^{-1}), methyl heptadecanoate (200 µg mL^{-1}), cis-10-heptadecanoic acid methyl ester (200 µg mL^{-1}), methyl stearate (400 µg mL^{-1}), trans-9-elaidic acid methyl ester (200 µg mL^{-1}), cis-9-oleic acid methyl ester (400 µg mL^{-1}), methyl linolelaidate (200 µg mL^{-1}), methyl linoleate (200 µg mL^{-1}), methyl arachidate (400 µg mL^{-1}), methyl γ-linoleate (200 µg mL^{-1}), methyl cis-11-eicosenoate (≤200 µg mL^{-1}), methyl linolenate (200 µg mL^{-1}), methyl heneicosanoate (200 µg mL^{-1}), cis-11,14-eicosadienoic acid methyl ester (200 µg mL^{-1}), methy behenate (400 µg mL^{-1}), cis-8,11,14-eicosatrienoic acid methyl ester (200 µg mL^{-1}), methyl erucate (200 µg mL^{-1}), cis-11,14,17-eicosatrienoic acid methyl ester (200 µg mL^{-1}), cis-5,8,11,14-eicosatetraenoic acid methyl ester (200 µg mL^{-1}), methyl tricosnoate (200 µg mL^{-1}), cis-13,16-docosadienoic acid methyl ester (200 µg mL^{-1}), methyl lignocerate (400 µg mL^{-1}), cis-5,8,11,14,17-eicosapentaenoic acid methyl ester (200 µg mL^{-1}), methyl nervonate (200 µg mL^{-1}), cis-4,7,10,13,16,19-docosahexanoic acid methyl ester (200 µg mL^{-1}) in dichloromethane.

The LC grade hexane and methanol were purchased from Merck (Darmstadt, Germany), and chloroform of the highest purity were purchased from Sigma-Aldrich (Praha, Czech Republic).

3.2.9.2.1.2 PREPARATION OF STANDARD SOLUTIONS

Calibration curves were made by direct dilution of the certified reference material based on the concentration of each component in the analyzed samples. The dilutions made were generally at 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 in dichloromethane.

The analytes found in Arabidopsis seeds were methyl palmitate (C16:0), methyl palmitoleate (C16:1), methyl gondoic acid (C20:1), methyl linoleate (C18:2), methyl stearate (C18:0), *cis*-9-oleic acid methyl ester (C18:1), *cis*-11,14-eicosadienoic acid methyl ester (C20:2), methyl arachidate (C20:0), methyl heneicosanoate (C21:0), and methyl erucate (C22:1).

3.2.9.2.2 METHODS

3.2.9.2.2.1 EXTRACTION METHOD PROCEDURE

The determination of fatty acids in Arabidopsis seeds was carried out following the method previously described by Carvalho and Malcata (2005)[370] with some minor modifications. The seeds from six plants per variant were homogenized and dried using a mixer-mill from Retsch Ceská Republika Verder (Praha, Czech Republic). 20 mg of Arabidopsis seeds were placed into an eppendorf vial of 1.5 mL and dropped in 1 mL of extraction mixture of chloroform/methanol ($CHCl_3$/MeOH, 2:1, % v/v) and sonicated for 10 min. The samples were centrifuged at 14,500 rpm for 2 min, and the solvent was transferred into a new eppendorf vial. After that, the solvent was evaporated at reduced pressure at 35ºC for 40 min, and then, 500 µL of 0.5 M of sodium methoxide (NaOMe) in methanol was added. The mixture was vortex 5 s and left 5 min at room temperature. The mixture was extracted twice with 500 µL of hexane and evaporated under reduced pressure at 35ºC for 5 min. Finally, the residues were constituted with 1 mL of labeled hexane and analyzed by gas chromatography coupled to mass spectrometry (GC-MS). All measurements were made in triplicate.

3.2.9.2.2.2 INSTRUMENTAL ANALYSIS

The assay of resulting fatty acid methyl esters (FAMEs) was carried out with a gas chromatography GC 1890 coupled to mass spectrometry method on the Agilent MSD 5975C series II system (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved on a fused silica HP-5MS UI column (30 m x 0.25 mm x 0.25 µm) from Agilent Technologies (Santa Clara, CA, USA) and helium carrier gas with a flow rate of 1.1 mL min^{-1}.

The elution consisted of a temperature ramp programmed at 120ºC for 3 min, 5ºC/min to 180ºC for 10 min, 3ºC/min to 220ºC for 3 min, and 2ºC/min to 250ºC for 5 min, and a post-run of 5 min was set at 310ºC. The injector and detector temperatures were 250ºC and 280ºC, respectively. Ionization was performed in an electronic impact (EI) mode at 70 eV. Identification was performed comparing retention times and mass spectra with authentic standards, as shown in *Fig. 3.7*.

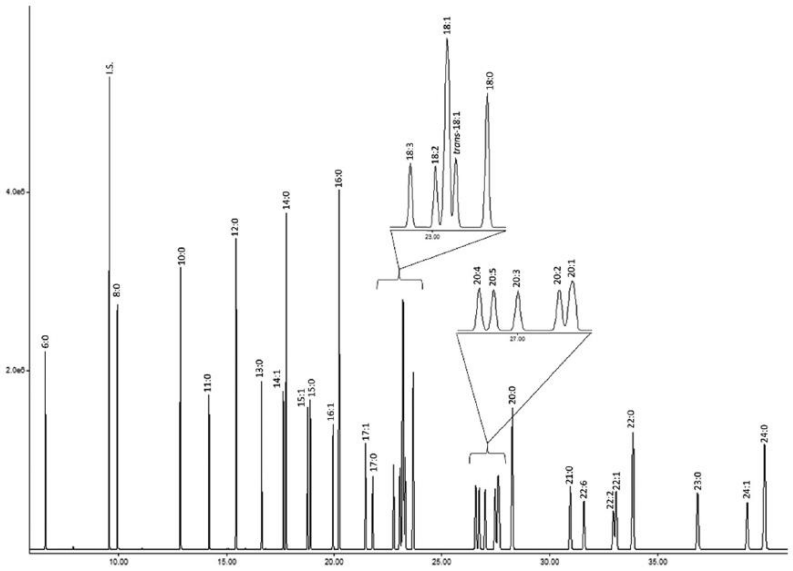


Figure 3.7. *Fatty acid standard reference material (CRM) chromatogram certified in accordance with ISO 17034 and ISO 17025.*

3.2.10 OSMOLYTES QUANTIFICATION

3.2.10.1 ORGANIC ACIDS QUANTIFICATION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO DIODE ARRAY DETECTOR (HPLC-DAD)

Forty-eight Arabidopsis seedlings were characterized by the developed analytical methodology HPLC-DAD described in section 3.7.1.2 with slight modifications.

For organic acids extraction in Arabidopsis samples, 5 mg of each lyophilized sample was used and mixed with 500 µL of 0.1 mM sodium hydroxide. The mixture was kept in the dark at 4ºC for 1 h and, after that, sonicated for 10 min. Then, 60 µL of (5% w/v) sodium chloride was added and stirred for 5 min. The samples were washed with 75 µL of Milli-Q water and centrifuged at 8,000 rpm for 10 min. Finally, the residues were acidified until pH 2.7 with H_3PO_4 (50% v/v). After that,

the samples were filtered through 0.22 µm PVDF syringe filters from Teknokroma (Barcelona, Spain). The extraction procedure described was carried out in triplicate.

3.2.10.2 FREE AMINO ACIDS QUANTIFICATION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY (UPLC-MS/MS)

3.2.10.2.1 MATERIALS

3.2.10.2.1.1 STANDARD AND REAGENTS

Individual high purity AAs of Asp, Glu, Gln, Arg, Ala, Asn, GABA, N-acetylornithine (AcOrn), 2-aminoadipic acid (AAA), BABA, Ala, γ-cystine (Cis), citrulline (Citr), cysteine (Cis), glycine (Gly), histidine (His), hydroxyproline (Hpr), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), ornithine (Orn), phenylalanine (Phe), Pro, serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), S-AdenosinHomocisteine (SAH), *L*-Homoarginine (HomoArg), and valine (Val) were purchased from Sigma-Aldrich (Czech Repulic). γ-aminobutyric acid-2,2,3,3,4,4-d_6 (GABA IS), leucine-2,3,3,4,5,5,5,5′,5′,5′-d_{10} (Leu IS), glutamic acid-2,3,3,4,4-d_5 (Gln IS), glutamine-2,3,3,4,4-d_5 (Glu IS), proline-$^{13}C_5$,^{15}N (Pro IS), and threonine-$^{13}C_4$,^{15}N,2,3,4,4,4-d_5 (Thr IS), purchased from Sigma-Aldrich (Praha, Czech Republic) were used as internal standards for amino acids quantification by HPLC-MS/MS.

The LC-MS grade ACN used in the mobile phases were purchased from Merck (Germany). Formic acid (FA), absolute EtOH, and HCl were of the highest purity available and purchased from Sigma-Aldrich (Praha, Czech Republic).

Ultra-high purity water was obtained from tap water pre-treated by Elix20 reverse osmosis and purified by the Milli-Q filtration system from Millipore (Bedford, USA). Moreover, mobile phases were filtered through 0.22 µm Polytetrafluoroethylene (PTFE) filters from Millipore (Watford, Ireland).

3.2.10.2.1.2 PREPARATION OF STANDARD SOLUTIONS

Stock standard solutions (10 mM) of all AAs and internal standards were prepared by dissolving the analytes in 0.1 N of HCl, except Asn, Gln, and Gln internal standards dissolved in MiliQ water (Millipore, Germany). These stock solutions were kept at -20°C and were stable for two months of continuous use.

Calibration standards were prepared by mixing and diluting stock solutions in the mobile phase ranging from from 0.075 µM to 500 µM.

3.2.10.2.2 METHODS

3.2.10.2.2.1 EXTRACTION METHOD PROCEDURE

Cleaned Arabidopsis leaves were lyophilized (Martin Christ Beta 1-8 LDPlus lyophilizer (Sigma-Aldrich, Steinheim, Germany).) to complete dryness (dry sample) and homogenized in a mixer mill (Retch, Germany). Samples were stored at -20 °C until extraction.

Aliquots of 5 mg of dry tissue were weighed directly into a 2 mL vial, and 1 mL of solvent (50 % EtOH) was added. After sonication for 10 min, extracts were centrifuged at 14,500 rpm for 5 min at 25°C, and supernatants were collected in a new vial. 200 µL of extracts were evaporated to dryness and redissolved in 50 µL of the mobile phase.

3.2.10.2.2.2 INSTRUMENTAL ANALYSIS

Target analytes in Arabidopsis seedlings were analyzed with an ultra-high performance liquid chromatography UPLC-MS/MS; Nexera X2 UHPLC coupled with a mass spectrometry detector MS-8050 (Shimadzu Handels GmbH, Kioto, Japan). Aliquots of 2 µl of each mixture and standard were injected, and the chromatographic separation was performed on Acquity UPLC BEH Amide

column (50 × 2.1 mm; 1.7 µm particle size) with its subsequent precolumn installed in an oven at 40ºC and eluted at a flow rate of 0.4 mL min^{-1}.

Mobile phase A consisted of a mixture of 20 mM ammonium formate (pH 3.0) and 0.2% of formic acid in ACN (phase B). Binary gradient consisted of 12 min programmed as follows; 0.0-2.0 min, 90% (B); 2.0-4.0 min, 85% (B); 4.0-6.0 min, 65% (B); 6.0-7.0 min, 55% (B); 7.0-7.2 min, 55% (B); 7.2-7.3 min, 90% (B) and 7.3-12.0, 90% (B). A diverter valve was used to automatically divert salt and buffers coming from the mobile phase to waste at the beginning of the run and highly hydrophobic compounds that are not of interest at the end of every run.

The mass spectra for all analytes, both non-derivatized compounds and benzoylated polyamines, were obtained via electrospray ionization in a positive mode under the following operating parameters: capillary voltage -3 kV; interface voltage 4 kV; interface temperature 300°C, heating, and drying gas flow 10 L min^{-1}; nebulizing gas flow 3 L min^{-1}. The multiple reaction monitoring (MRM) transitions are summarized in **Annex VII** (*Table VII.1*). The identification of analytes was performed by comparing retention times and MRM transitions and relative intensities with authentic standards, while the isotope dilution method was quantified.

3.2.10.2.2.3 ANALYTICAL METHOD EVALUATION

In addition, the LC-MS/MS analytical methodology was validated for linearity, limits quantification (LOQs), precision (interday and intraday precision), reproducibility, and stability following the ICH guidelines (ICH, 2006)[371], and some published analytical studies (Zhou *et al.* 2013; Tarkowska *et al.* 2016; Wen *et al*. 2019)[372-374].

According to the isotope dilution analysis method, calibration curves were constructed from peak areas of the reference standards of amino acids and biogenic amines versus their concentrations (Jonckheere *et al.* 1983)[375]. Each calibration curve was performed with ten concentrations in triplicate. The LOQs for each analyte were determined at signal-to-noise ratios (S/N) of 10.

3.3 STATISTICAL ANALYSIS

Data analysis of *Vitis vinífera* L. was performed using RStudio (R Software version 4.1.0) using packages *agricolae, ggplot2, factoextra* and *pls*. Data were normalized for the subsequent analysis. Three-way ANOVAs were used for LWC, yield-related parameters, and WUE followed by Duncan's tests for multiple comparisons. For the metabolites, multiple four-way ANOVAs including treatment (control *vs*. compound), time (sampling time), plot, and the year (2018 or 2019) as factors followed by Duncan's tests for multiple comparisons were performed, using Bonferroni corrections for multiple testing. Partial least squares discriminant analysis (PLS-DA) was performed using the SIMPLS algorithm and taking treatment (control vs. one of the others) as the dependent (binary) variable. PLS biplots were displayed.

The phenotyping and biochemical dataset were analyzed using a multi-ANOVA analysis. Multivariate statistical analysis was also performed, and principal component analysis (PCA) and matrix correlation based on Pearson correlations performed using RStudio (R Software version 4.1.0) through the packages *corrplot* and *ggplot2*.

Some parameters such as FW, DW, biomass, RWC, green pixels, FTemp, QY_max, Fv/Fm_Lss, NPQ_Lss, Rdf_Lss, A, gs, Ci, E, WUE and total metabolites, were analyzed together at two different times for the study; the first time after the first foliar biostimulant application to evaluate the fast response to the biostimulant application and under moderate drought stress, and the second at the end of the phenotyping protocol when plants were under severe drought stress.

In addition, for multi-ANOVA analysis, the area under the curve (AUC) was used in the photosynthetic parameters corresponding to maximum photosystem II (PSII) quantum yield (QY_maxAUC) and steady-state PSII quantum yield (QY_LssAUC), respectively, a mathematical integration calculated from the sum of the trapezoids, where a single value of the curve is calculated for each experimental unit. Based on this value, a statistical analysis like ANOVA compares the differences between the curves (*Eq. 3.10*).

$$\mathrm{AUC} = \sum_{i=1}^{n-1} \frac{(Y_i + Y_{i+1})}{2} \cdot (t_{i+1} - t_i) \qquad \textbf{(Eq 3.10)}$$

From morphological parameters and soil water content in the different watering conditions, a one-way analysis of variance (ANOVA) with post hoc Tukey's Honest Significant Difference (HDS) test ($p<0.05$) was performed.

Chapter IV: Biostimulant uses to improve production quality of *Vitis vinífera* L. against water stress

4.1 INTRODUCTION

Viticulture is currently affected by climate change. Many vineyards are recording drastic increases in air temperature and significant changes in rainfall distribution, especially during the growing season[32,376].

Drought is a combination of multiple stresses; water, light, and temperature[377], reducing the absorption of water ability from the soil[378]. The main impact of reduced water availability on grapevine physiology is the prevention of vegetative and reproductive growth and decreased canopy assimilation and transpiration rate[379,380].

In general, mild water deficits promote berry quality in red varieties[381]. However, under severely limited water conditions, leaves experience irreversible photoinhibition and yellowing, leading to leaf abscission[377]. In addition, the berry size is reduced due to the water restriction of plants, increasing the weight ratio of the skin to the pulp and, consequently, the accumulation of different compounds in the berry skins[382].

It has been shown that extracts based on seaweed AN applied to vines promote plant growth, fruit yield, and quality[172,178,383,384], since they improve the synthesis of anthocyanins[176], the water status of leaves by promoting the thermoregulation, reduced stomatal sensitivity in *Vitis vinifera* L.[387], improve hydraulic conductance and maintain optimal gas exchange[176].

Most commercial extracts from brown algae are composed of a wide range of organic and inorganic components[174], such as micro/macronutrients, osmolytes, AAs, proteins, vitamins, and polysaccharides[174].

There are few reviews in literature dealing with the impact of AN on vines under water stress since most reports on the use of these extracts in vines are focused on their effects on physiology, oenological parameters and/or productivity such as fruit set, leaf size, weight and size of berries, soluble solids, pH, total acidity, total anthocyanins, and total polyphenol index on nitrogen content in must or sugar content in leaves[114,177,378,388-392]. Most authors report few differences in the oenological parameters of grapes under experimental field conditions. Gutierrez-Gamboa *et al.* (2018)[393] confirmed the foliar application of AN on vines increased the AAs content in grapes,

although the most impact factor that affected the variability of nitrogen concentration in the must is the climatic conditions of the season[390]. Similarly, Shukla *et al.* (2019)[394] published the positive effect of AN extracts under water scarcity by modulation of plant hormonal signaling and the biosynthesis of antioxidants and/or osmotically active compounds such as flavonoids, carotenoids, betaines, or carbohydrates. Likewise, other parameters such as water balance, gas exchange, and photosystem II efficiency play an essential role in the tolerance mechanisms to water stress in grapevine[46,176,178,379, 387,390].

The application of AN extracts increases the solutes that affect the plant osmoregulation capacity during water stress[173,395], providing precursors that protect leaf tissues against reactive oxygen species formed during stress[396]. This broad efficacy can be attributed to the high concentration of AAs present in AN extract, which can act as compatible solutes under osmotic stress[173,395], and the presence of secondary bioactive metabolites such vitamins or their precursors[397] that protect plant tissues against ROS induced by stress[398]. Carbohydrate content in leaves also helps the turgor pressure regulation to maintain favorable assimilation rates under stress conditions, prevent the photosystem II damages, and promote osmotic adjustment together with organic ions[398-400], whose content varies during the growth stages according to the water status[401,402]. In general, more carbohydrates indicate higher relative water content in leaves[398].

Although it was reported that the high content of AAs presented in the biostimulant extracts has positive effects on the vine[403], this content could not be sufficient to improve plant growth by applying them[389]. Therefore, the physiological mechanism of action is still not precise, and additional studies are still needed to clarify the mechanism and the dynamics of actions of various metabolites after foliar biostimulants application.

4.2 OBJECTIVES

The main objective was to determine the effect of the foliar application with three commercial biostimulants, two based on AN marine algae and one rich in AAs, on the quality and quantity of plant productions and to define their mechanism/mode of action on *Vitis vinifera* L. affected by the climate change through the evaluation of the plant water balance and specific modification of endogenous metabolites. For that purpose, it is necessary to achieve the following partial objectives:

- Study different physiological parameters such as environmental changes, leaf water relations, and vineyard productive parameters and survival.
- Determine and quantify primary metabolites (carbohydrates, AcOrg, and AAs) through different analytical methodologies based on HPLC coupled to index refraction detector (RIS), photodiode array detector (DAD) and fluorescence detector (FLD).
- Estimate their interconenections as active osmolytes of tolerance against stress.
- Screen the biostimulant mechanism/mode of action through the osmotic or physiological adjustment induced by the foliar application treatments in leaves, grapes, and musts throughout the vine's vegetative and reproductive cycle.

4.3 MATERIALS AND METHODS

4.3.1 PLANT MATERIAL, GROWTH CONDITIONS AND EXPERIMENTAL DESIGN

Vitis vinifera L. vines of Tempranillo variety planted in 1984 on 110 Ritcher rootstock were used. The experimental design, described in section 3.1.2 (Chapter III), consisted of three commercial biostimulants base don AN and AAs used to treat the vineyard for two consecutive vintages (2018 and 2019) to deal with the wáter stress suffered in recent years in the DOCa Rioja.

The respective samplings carried out throughout this study are described in sections 3.1.3.1 and 3.1.3.2 for leaves, grapes, and musts.

4.3.2 WATER STATUS DETERMINATION

LWC (%) was determined according to *Eq. 3.1*, described in section 3.1.4 (Chapter III) in leaves samples collected before the second biostimulant foliar applications (T0B) and 24 h after the second biostimulant foliar applications (T24B).

4.3.3 PRODUCTION PARAMETERS

Before the maturity period (TV) and the optimal maturity (TF) the number of bunches per vine (n), yield (kg/vine), average weight of bunches (g), bunch length (cm) and berry diameter (mm), were studied as described in section 3.1.5.1 (chapter III).

Likewise, the survival of vines was controlled during both vintages, following the *Eq. 3.2* described in section 3.1.5.1 of chapter III.

4.3.4 CLIMATE DATA, PHENOLOGICAL STAGES AND WATER USE EFFICIENCY (WUE)

Climatic conditions during both vintages (2018 y 2019) were recorded thanks to the data provided by a meteorological station near the vineyard including the maximum temperature, minimum temperature, and daily rainfall (data shown in *Table II.1*, **Annex II**). The phenological stages as BB, BL, V and M were recorded through direct observation of the vineyard, as shown in *Table II.2* of **Annex II**. This data was used to calculate the theoretical water status of the vineyard during the growing seasons, through the reference (ET_0) and effective (ET_c) crop evapotranspiration, according to *Eq. 3.3* and *Eq. 3.4* described in section 3.1.5.2 of chapter III.

Finally, production WUE was determined and calculated as the relation between the accumulated ET_c from budbreak to maturity and the final yield of the vineyard (kg ha^{-1}).

4.3.5 OSMOLYTES QUANTIFICATION

Carbohydrates (Fruc, Gluc, Sac and Malt), AcOrg (Oxal, Tart, Mal, Acet, Cit, Succ, and Fum), and free AAs (Asp, Glu, Asn, Gln, Arg, Ala, GABA, BABA) in one hundred leaves samples of *Vitis vinifera* L. from samplings carried out before the first biostimulant foliar application (T0A), 24 h after first biostimulant foliar application (T24A), before the second biostimulant foliar application (T0B), 24 h before the second biostimulant foliar application (T24B) and the optimal maturity (TF), as well as in 40 must and grape from samplings carried out before maturity (TV) and at the optimal maturity (TF) as described in sections 3.1.6.1, 3.1.6.2 and 3.1.6.3.

For that purpose, three analytical methodologies have been validated for each analytes family, as described in sections 3.1.6.1, 3.1.6.2, and 3.1.6.3, based on high-performance liquid chromatography (HPLC) coupled to an index refraction detector (RID), photodiode array detector (DAD) and fluorescence detector (FLD) respectively.

4.4 RESULS

4.4.1 WATER STATUS DETERMINATION

Before (T0B) and after the second application (T24B), the plant's water balance calculating the LWC (%) was studied to clarify the ambient and the application effect (*Fig. 4.1.*).

The application was performed during bloom. Before the application, LWC was lower in the plants in 2019 than in 2018 (*Fig. 4.1A.*), most probably due to the high temperatures recorded in this period (*Fig. 4.1B.*). In general, the foliar application negatively impacted the LWC, reducing the values in both seasons (2018 and 2019). Only those plants sprayed with BF or GT improved their LWC in 2019 (*Fig. 4.1A.*). Subplots A and B were analyzed separately to better understand the biostimulant effect on the LWC.

The ratio between the LWC of the plants after (T24B) and before (T0B) the second foliar application was represented for better visualization of the results (*Fig. 4.1B.*). Again, an apparent

negative impact of the foliar application in the LWC was visible in all the treatments in 2018, including the controls (*Fig. 4.1B.*). The LWC was lower in those plants from subplot A than from B.

The same happened in 2019. However, in this case, the plants sprayed with BF increased the LWC at T24B compared to the same plants at T0B in both A and B subplot (*Fig. 4.1B.*). The plants sprayed with GT also tended to maintain or increase the LWC in A or B, respectively, after application (*Fig. 4.1B.*). Altogether, the results showed a positive effect of certain biostimulants when applied at bloom in wine plants subjected to high temperatures.

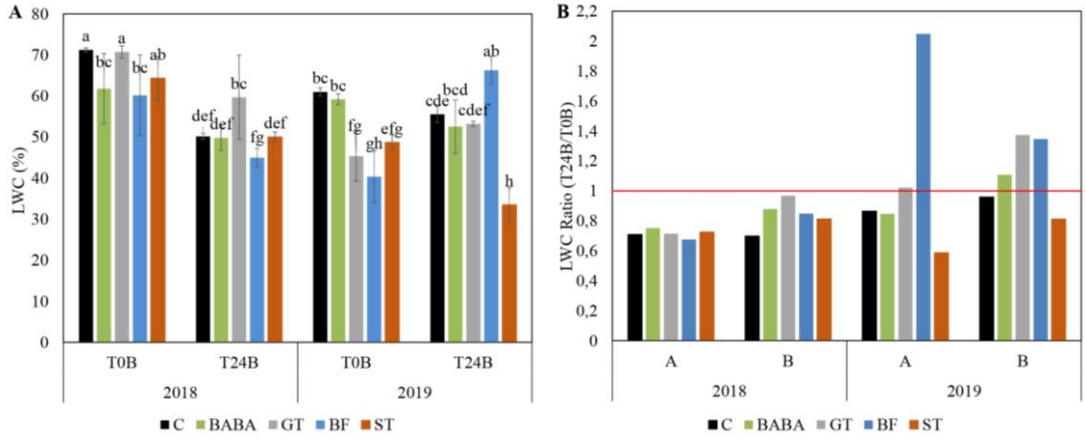


Figure 4.1. *Leaf water content (LWC, %) in Vitis vinifera var. Tempranillo before (T0B) and after (T24B) the second foliar application with distilled water (C), BABA or three commercial biostimulants [Greetnal (GT), Basofoliar (BF) or SoilExpert (ST)] in 2018 or 2019 (A). Ratio of the LWC between T24B and T0B for the two subplots and seasons (B).*

4.4.2 CLIMATE DATA, PHENOLOGICAL STAGES AND WATER-USE EFFICIENCY

The precipitation and temperature of the growing season were followed to understand the climate's impact on our vineyard. *Fig. 4.2A.* shows that higher precipitations occurred in 2018 during the budbreak but then reduced until maturity. Contrarily, the T_{Max} and T_{min} increased. These climatic conditions induced a high increase in the ET_c, especially at bloom and to less extent at veraison. Consequently, the vineyard presented lower WD in these two developmental stages and reached the lowest values at maturity (*Fig. 4.2B*).

A different profile was observed in 2019. This year was dried, and the precipitations were lower than in 2018 for all development stages analyzed, especially at veraison (*Fig. 4.2C.*). Only higher precipitations were registered at maturity. Additionally, at bloom, T_{Max} reached values of 40.5ºC, 7ºC more than in 2018 (*Figure 4.2C.*). A low pluviometry at veraison and high temperatures at bloom increased the ET_c and reduced the WD in the vineyard, showing that the plants suffered a higher restriction of water available for the crop than in 2018 during very relevant developmental stages.

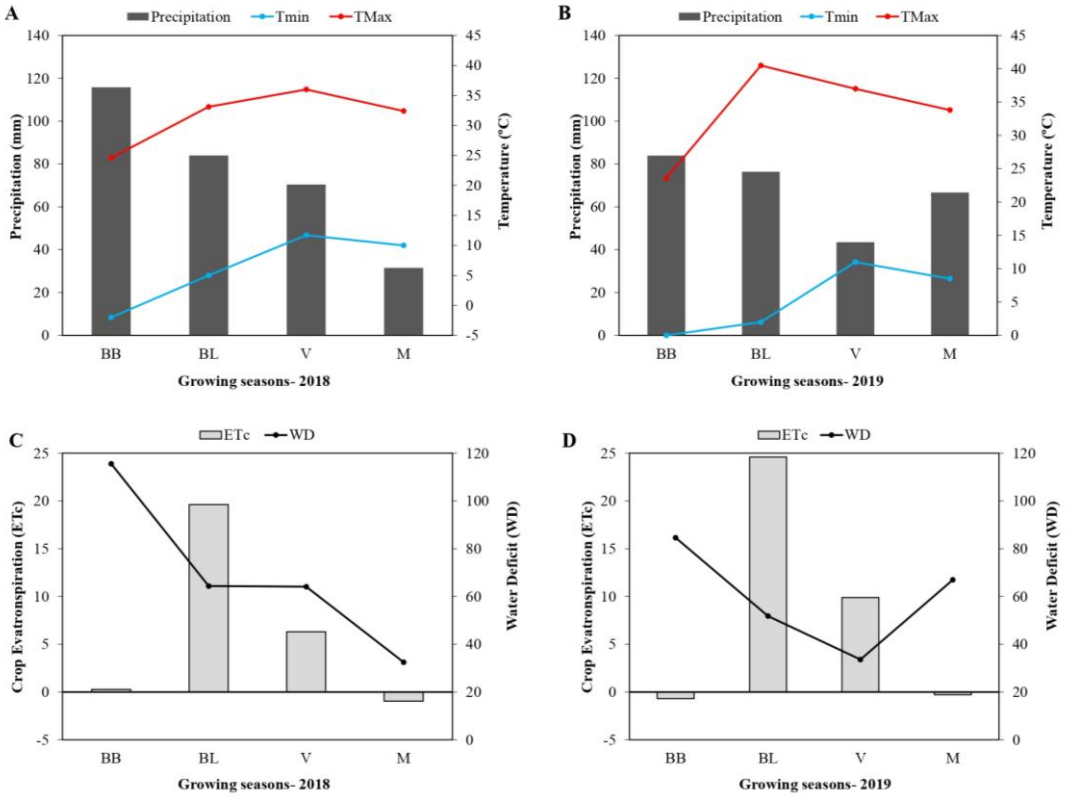


Figure 4.2. *Precipitation, maximal temperature (Tmax) and minimal temperature (Tmin), and the calculated crop evapotranspiration (ETc) and water deficit (WD) of the vineyard in 2018 (A and B) and 2019 (C and D).*

Finally, as the next step, WUE was calculated. As a result, we observed that biostimulant application modified the water use efficiency related to production, and only the plants sprayed with BABA significantly increased the WUE compared to the controls in subplot A in 2018 (*Fig. 4.3*).

The commercial substances did not change or decrease WUE (*Fig. 4.3*). The most considerable reductions always happened in subplot B, especially for the year 2019. Only the application of GT slightly improved the WUE in subplot B in 2018 (*Fig. 4.3*).

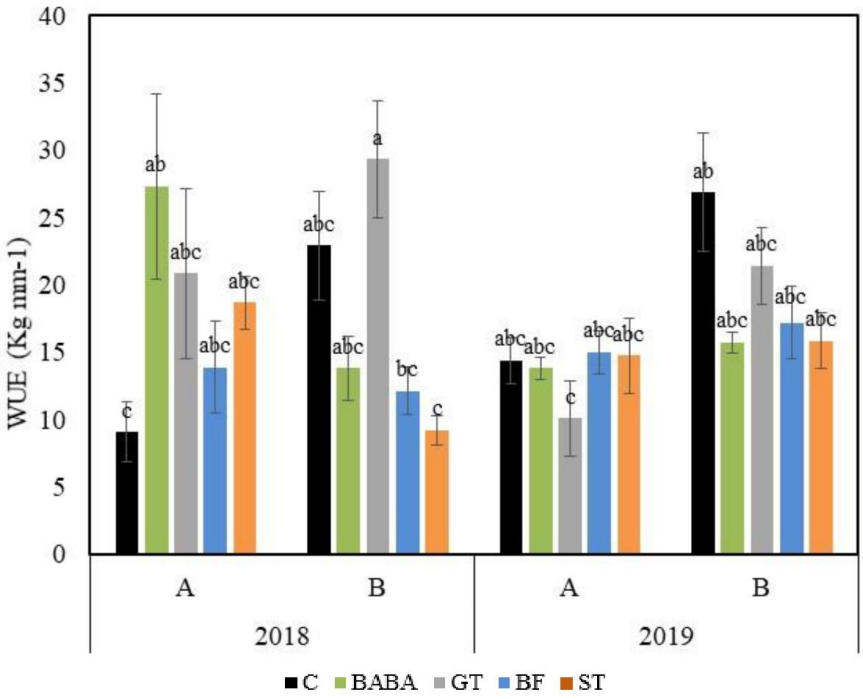


Figure 4.3. *Water-use efficiency (WUE, Kg mm^{-1}) in plants of* Vitis vinifera *var. Tempranillo from two subplots (A and B) sprayed with distilled water (C), BABA or three commercial biostimulants [Greetnal (GT), Basofoliar (BF) or SoilExpert (ST)] in the seasons 2018 or 2019.*

4.4.3 PRODUCTION PARAMETERS

At the end of each season, different parameters related to the yield and SV (%) were determined in the vineyard. Again, apparent differences were observed between subplots and years and the interaction of both (*Fig. 4.4*). No variations in the yield-related parameters were observed between treated and untreated plants in 2019 (*Fig. 4.4*).

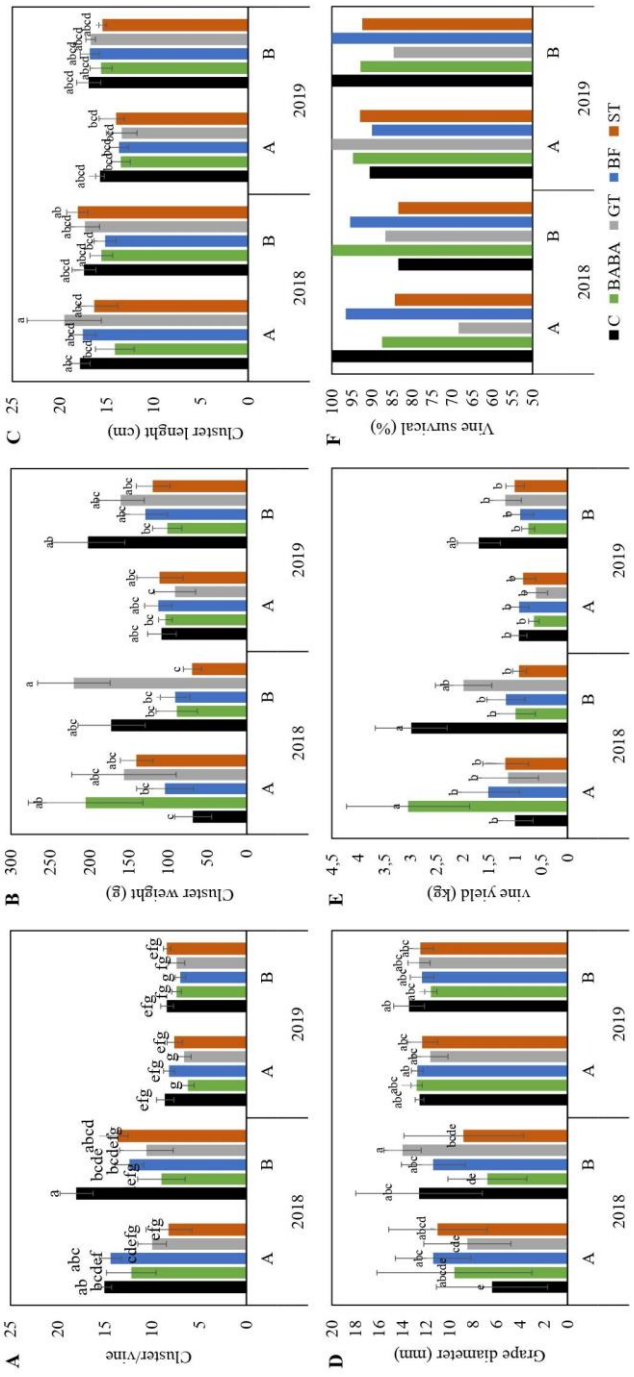


Figure 4.4. *Number of clusters per vine (A), cluster weight (g, B) and length (cm, C), grape diameter (mm, D), vine yield (Kg, E) and survival (%, F) in plants of* Vitis vinifera *var. Tempranillo from two subplots (A and B) sprayed with distilled water (C), BABA or three commercial biostimulants [Greetnal (GT), Basofoliar (BF) or SoilExpert (ST)] in the seasons 2018 or 2019.*

However, their foliar application modified the number of clusters and the dimensions of the grapes in 2018. For example, biostimulants reduced the clusters per plant in 2018, except for BABA and BF or ST in subplots A and B, respectively (*Fig. 4.4A*). BABA application enhanced the weight of the grapes and the final vine yield (kg) in subplot A but not in B, compared to controls (*Fig. 4.44B* and *4.4E*). One possible reason for the good results observed with BABA was a better fruit setting and the low incidence of diseases in the treated plants (*Fig. 4.5* and *Table I.1* and *Table I.2* of **Annex I**). Contrarily, BF and ST increased the grape diameter compared to untreated vines in subplot A in 2018.

The vine survival (%) in the old and low productive vineyards was also determined because the study was performed. No considerable effect on the plant survival was observed (*Fig. 4.4F*). The best results were observed when BABA was applied in subplots B in 2018 and A in 2019. Contrarily, the ST application reduced the survival in subplots A in 2018 and B in 2019 (*Fig. 4.4F*).

Altogether, we could conclude that biostimulant application improved the production under moderate but not severe stress conditions. Also, the efficiency of the biostimulants strongly depends on the interactive effect between the year (climatology) and the characteristics of the plot, and the non-protein amino acid BABA is the most promising biostimulant for plant production and survival.

To better visualize and simplify the biostimulant characterization, we used the PBC index developed by Ugena *et al.* (2018)[404]. This approach ended with a positive or negative value that permits the classification of the biostimulant. This index is calculated by the sum of all values represented in a parallel coordinate plot per variant. This value is the log2 of the ratio between the untreated and treated plants[404].

Four parallel coordinate plots were performed, two for the PBC index representing the plant physiology and survival for the season 2018 and 2019, and two additional ones with the production-related parameters (*Fig. 4.5*). As the main result, an adverse effect of the biostimulant application was observed in the vines located in subplot B (discontinuous lines, *Fig. 4.5*), which ended with negative values of PBC index (*Table 4.1*).

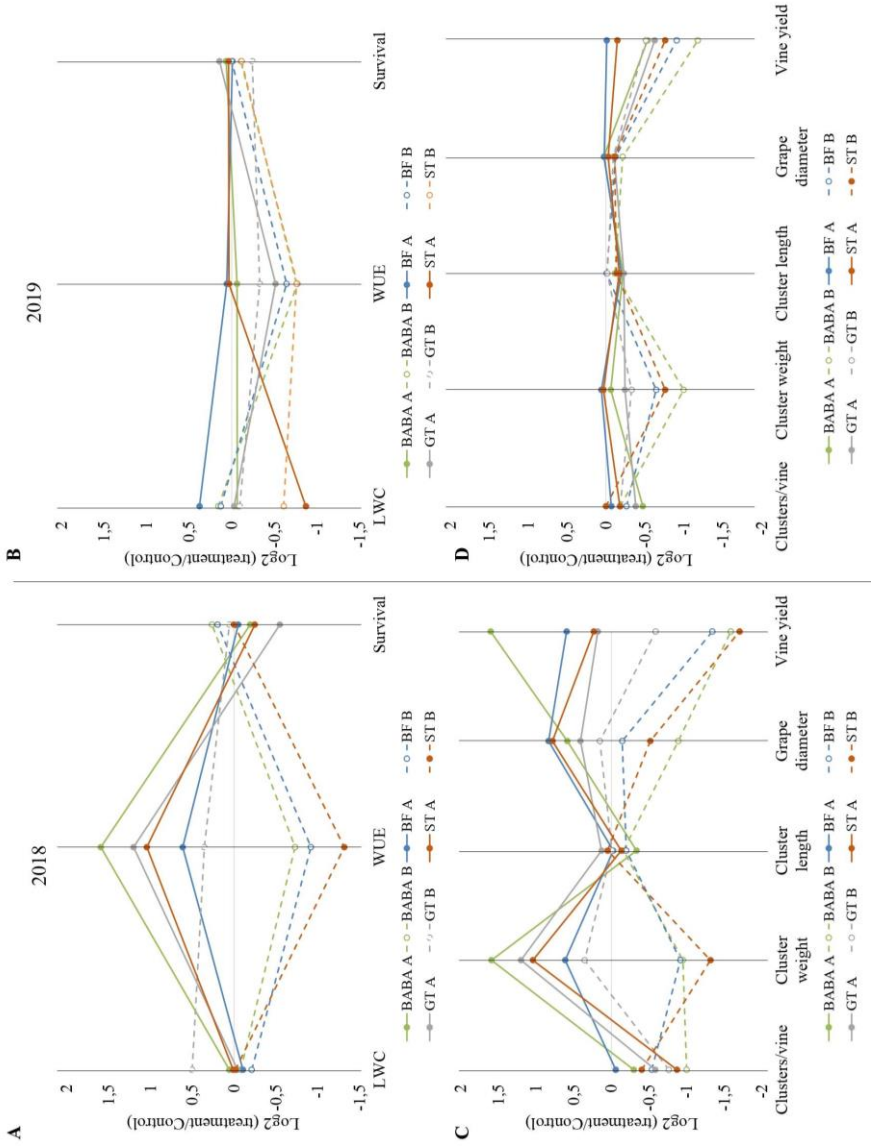


Figure 4.5. *Parallel plots representing the LWC and survival (A and B) and the production (C and D) as the log2 of the ratio between the treatment and control in plants of* Vitis vinifera *var. Tempranillo from two subplots (A and B) sprayed with distilled water (C), BABA or three commercial biostimulants [Greetnal (GT), Basofoliar (BF) or SoilExpert (ST)] in the season 2018 or 2019. Table representing the PBC for plant physiology and survival or production is in the lower panel.*

Only the use of GT improved plant physiology in 2018 (*Fig. 4.5A*). In subplot A the foliar application with biostimulants improved vine physiology and yield in 2018 but not in 2019, so all variants presented positive PBC index values (*Table 4.1*). No correlation was observed between the PCB index values for physiology and yield. However, the variants with positive values of PBC index for the physiology (mainly better water balance) ended with better yield or at least maintained it. Altogether, we could conclude that the application of biostimulants on vines improves final yield when they can induce a more efficient use of the water.

Table 4.1. *PBC index for plant physiology and survival or production yield parameters in pants of* Vitis vinifera *var. Tempranillo from two subplots (A and B) sprayed with distilled water (C), BABA or three commercial biostimulants [Greetnal (GT), Basofoliar (BF) or SoilExpert (ST)] in the season 2018 or 2019.*

Biostimulant	PBC index (Physiology-survival)				PBC index (Yield)			
	2018		2019		2018		2019	
	A	B	A	B	A	B	A	B
GT	0.61	0.91	-0.40	-0.66	1.33	-0.85	-1.13	-0.95
BF	0.46	-0.93	0.43	-0.51	1.96	-3.14	-0.12	-1.68
ST	0.80	-1.34	-0.80	-1.48	1.05	-3.89	-0.31	-1.77
BABA	1.46	-0.55	-0.06	-0.72	3.15	-4.59	-0.78	-2.50

4.4.4 OSMOLYTES QUANTIFICATION

The content of concrete-free AAs, AcOrg, and carbohydrates was performed in different matrices (leaves, grapes, and musts). Due to the complex experimental setup, we performed a multivariate statistical analysis based on PLS-DA for better visualization. This approach permitted the direct comparison between control and treatment, ending with 4 PLS-DA figures for each type of matrices.

As can be observed, the application of biostimulants altered the metabolite content in vines. In leaves, the distribution obtained by the PLS component 1 and 2 mainly separated the effect by season (2018 or 2019) and sampling time. The latest samplings (TF) reduced the GABA and BABA content in leaves in all plants (treated and untreated) (*Fig. 4.6* and *Tables VIII.7A-7B*, and *Tables VIII.16A-16B* of **Annex VIII**). However, some concrete responses were also observed. For example,

BABA-sprayed plants accumulated higher content of free AAs, especially Arg and Gln at TF (*Fig. 4.6A*), whereas GT-treated ones accumulated Mal and Oxal (*Fig. 4.6C* and *Tables VIII.4A-4B*, and *Tables VIII.13A-13B* of **Annex VIII**).

In grapes and must, the differences between treatments were more evident, but the content of metabolites among both matrices also differed (*Fig. 4.7*). As the most relevant result, we observed that the AcOrg were the main contributors to separating the treated and untreated variants. All treatments increased Fum, independent of the subplot and year (*Fig. 4.7 and Tables VIII.5, Table VIII.6, Table VIII.14, Table VIII.15* of **Annex VIII**). However, it only happened in grapes from BABA and BF-treated plants. The metabolite content of the GT and ST-treated plants distributed the response on the PLS-DA mainly due to the season (*Fig. 4.7E* and *4.7G*). The biostimulant treatment also reduced the sugar content, especially in musts, except GT (*Fig. 4.7A* and *4.7C*, and *Tables VIII.2* and *VIII.11* of **Annex VIII**). Altogether, the foliar application with biostimulants modifies the content of AcOrg and sugars mainly in the reproductive organs, like grapes (*Tables VIII.3, VIII.A, VIII.B, VIII.12 VIII.15A. VIII.B*. of **Annex VIII**).

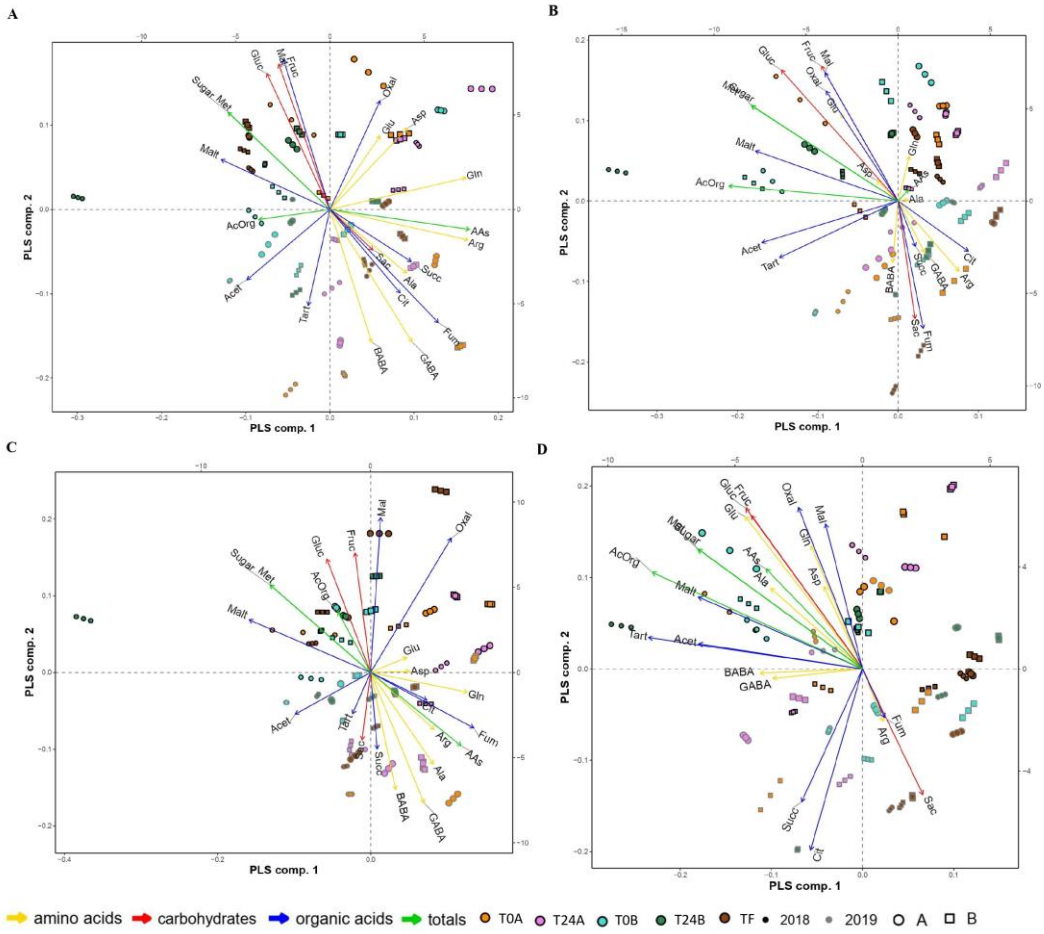


Figure 4.6. *Partial least squares discrimination analysis (PLS-DA) in leaves of* Vitis vinifera *var. Tempranillo from two subplots (A and B) collected before and after the first (T0A and T24A) and second (T0B and T24B) foliar application and at maturity (TF) in the seasons 2018 or 2019 with distilled water (C) vs BABA (A), distilled water (C) vs Basofoliar (BF) (B), distilled water (C) vs Greetnal (GT) (C), distilled water (C) vs SoilExpert (ST) (D). Free AAs; L-aspartic acid (Asp), L-glutamic acid (Glu), L-asparagine (Asn), L-glutamine (Gln), L-arginine (Arg), L-alanine (Ala), γ-aminobutyric acid (GABA), β-aminobutyric acid (BABA) and α-aminobutyric acid (AABA). Carbohydrates; D-(-)-fructose (Fruc), D-(+)-glucose (Gluc), sucrose (Sac), and maltose (Malt). AcOrg; oxalic acid (Oxal), L-(+)-tartaric acid (Tart), DL-malic acid (Mal), acetic acid (Acet), citric acid (Cit), succinic acid (Succ), and fumaric acid (Fum).*

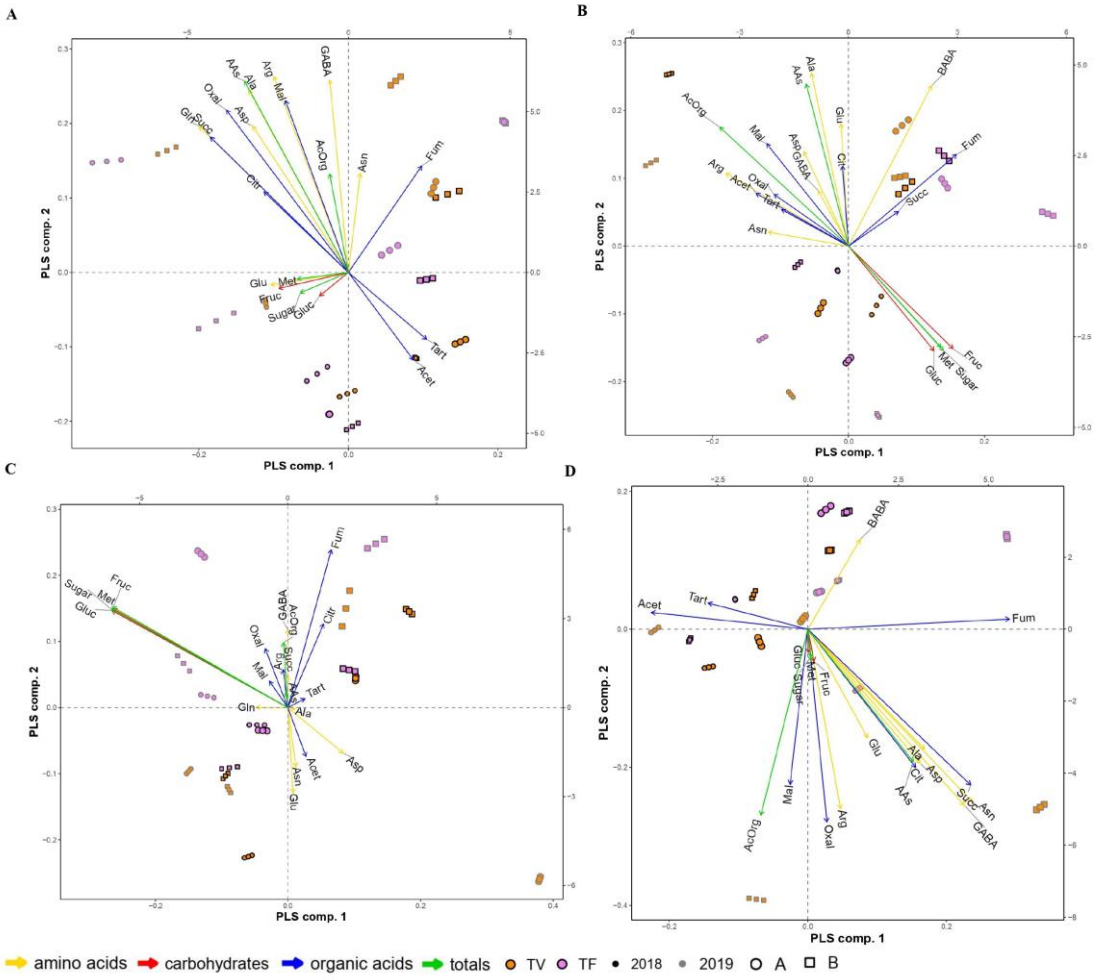


Figure 4.7. *Partial least squares discrimination analysis (PLS-DA) in grapes of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs BABA (A), must of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs BABA (B), grapes of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs Basofoliar (BF) (C), must of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs Basofoliar (BF) (D), grapes of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs Greetnal (GT) (E), must of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs Greetnal (GT) (F), grapes of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs SoilExpert (ST) (G), must of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs SoilExpert (ST) (H) from two subplots (A and B) collected before and after the harvest (TV and TF).*

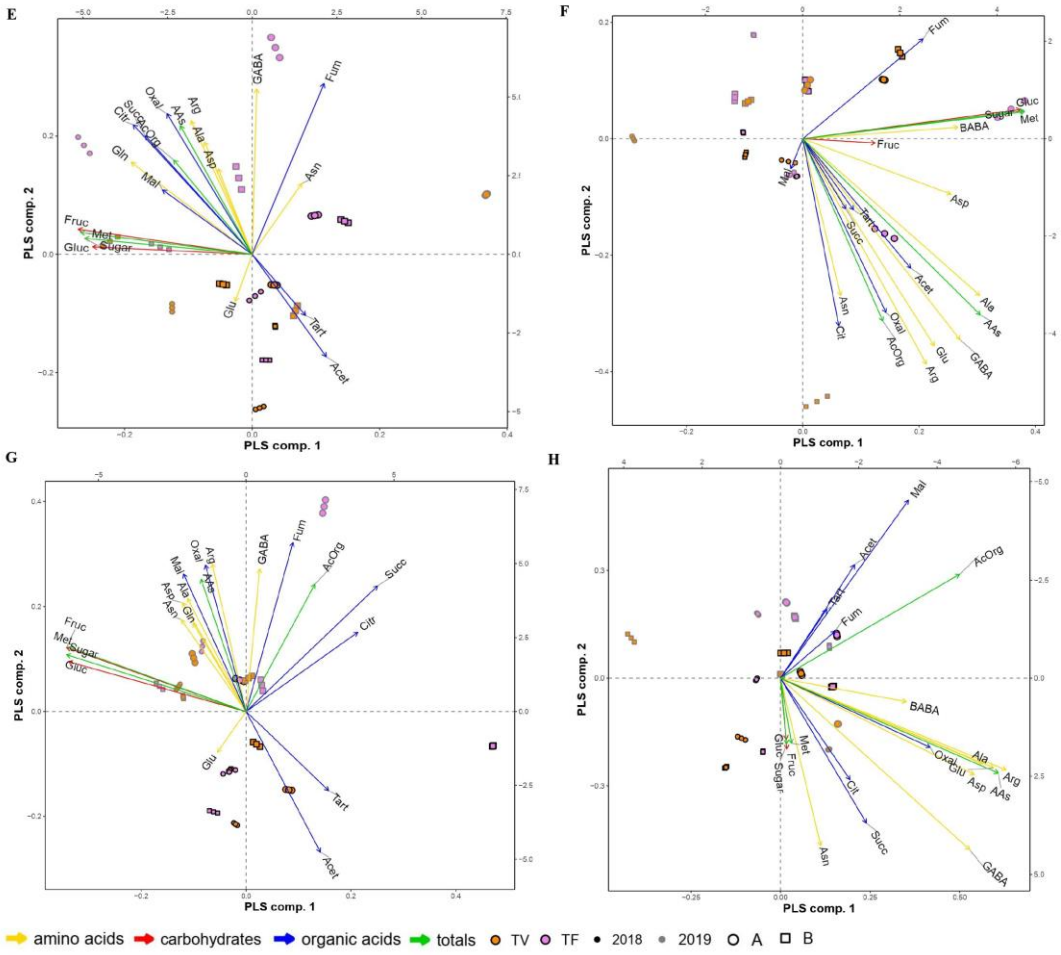


Figure 4.7 (Cont.). *Partial least squares discrimination analysis (PLS-DA) in grapes of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs BABA (A), must of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs BABA (B), grapes of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs Basofoliar (BF) (C), must of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs Basofoliar (BF) (D), grapes of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs Greetnal (GT) (E), must of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs Greetnal (GT) (F), grapes of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs SoilExpert (ST) (G), must of* Vitis vinifera *var. Tempranillo sprayed with distilled water (C) vs SoilExpert (ST) (H) from two subplots (A and B) collected before and after the harvest (TV and TF).*

As the final step, we performed a principal component analysis to compare the content of the studied metabolites in the commercial products used (*Table VIII.19* of **Annex VIII**). Again, different profiles were observed, including between GT and ST, biostimulants based on the seaweed *Ascophyllum nodosum* (*Fig. 4.8*). Whereas GT presented the highest Gln and Acet values, ST contained more Fum, Tart, and Oxal. BF presented the highest amount of free AAs and the organic acid Mal (*Fig. 4.8*). The differences in the metabolic profiles could also explain the specificities observed in the plant response.

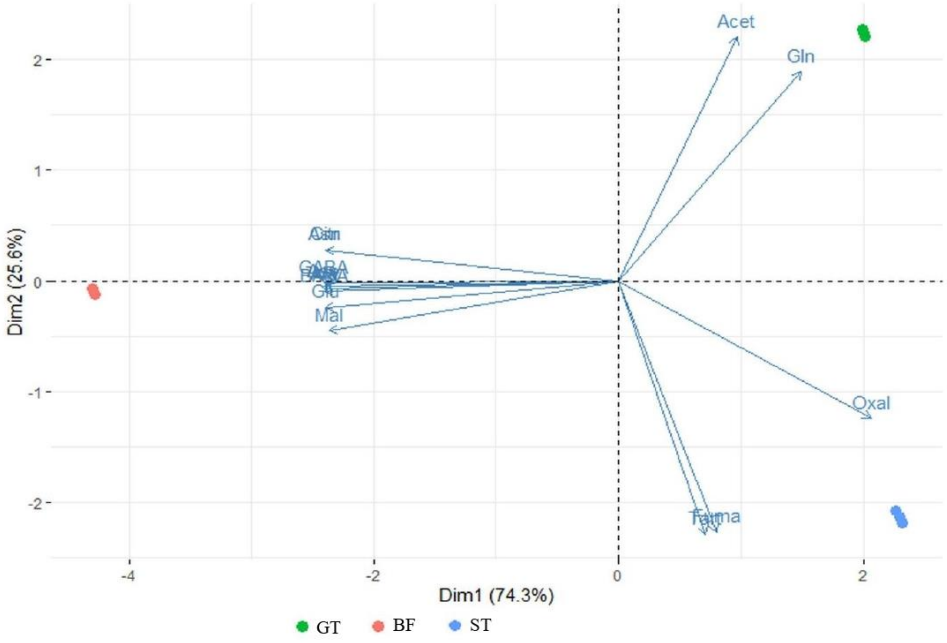


Figure 4.8. *Principal component analysis (PCA) of the metabolite content in three commercial biostimulants [Greetnal (GT), Basofoliar (BF) or SoilExpert (ST)].*

4.5 DISCUSSION

The use of biostimulants in grapevines is one of the most significant interests of wine producers to reduce the use of chemicals that negatively affect plant and human health[135,405,406]. They reported that biostimulants based on simple molecules or more complex substances could help the plant deal with biotic and abiotic stress. Biostimulants have been described to improve stress resilience mainly

by increasing photosynthesis, modifying metabolism, and activating the antioxidative response of the plants. The biostimulants based on *Ascophyllum nodosum* are not an exception, as described by Salvi et al. (2019)[46], Taskos et al. (2019)[391], Samuels et al. (2022)[406], and Frioni et al. (2019)[407]. *Ascophyllum nodosum*-based biostimulants can improve the plant performance and the yield and quality of plants subjected to different stress conditions[388,407]. We wanted further to characterize the biostimulant mechanism of action in our work. We compared the use of three commercial substances, one amino acid-rich formulation and two *Ascophyllum nodosum* extracts, and the non-proteinogenic amino acid BABA as a small molecule based-biostimulant on an old vineyard highly affected by the climate change. In general, the climatology of the year and the vine plant location (subplot) highly condition the effectiveness of the biostimulant application, especially in the WUE and yield-related parameters. However, the metabolite content of the berries (grapes) and musts changed due to the biostimulant treatment in a similar manner for the two studied seasons.

As mentioned above, the application of biostimulants in vineyards has been related to better plant's physiology under stress conditions. In our work, the biostimulants treated plants also improved the LWC and WUE (*Fig. 4.1* and *4.3*). However, the location and the year highly influenced the plant response. Whereas biostimulants enhanced LWC during the driest season (2019), WUE was improved only in subplot A in 2018.

Using the PBC index to compare the results related to plant physiology and yield (*Fig. 4.6*), we could conclude that WUE but not the LWC condition plant production, so only the biostimulants treated vine plants from subplot A in 2018 presented positive values. One possible explanation is that the high temperatures are the most limiting factor in grapevine[408]. We observed that the maximal temperatures in 2019 during the relevant phenological stages like BL and V were much higher than in 2018. BABA was the most efficient biostimulant, improving vine yield mainly by berries with higher diameter. One of the reasons could be the low incidences of diseases observed in the BABA-treated plants in 2018 (*Fig. 4.9* and *4.10* and *Fig. I.1* to *I.10* of **Annex I**). BABA has been well documented as a predominant priming elicitor for effective resistance induction and is desirable for disease management in agricultural fields[202,409]. Its application has also been used to induce resistance in grapevine (*Vitis vinifera*) against downy mildew[192] or to increase the biotic resistance in postharvest grapes[410]. However, the positive effect of BABA was not visible in subplot B in 2018. In this regard, Kocsis et al. (2018)[411] observed that BABA can also inhibit flower fertility

and, hence, berries per cluster and weight, pointing to the interaction between BABA treatment and the environmental pressure as the determinant factor for the positive or negative response of the plant. Finally, it is also worth mentioning that WUE has been described as the capacity of a crop to produce biomass per unit of water evapotranspiration and the major component of yield[412]. We could conclude that biostimulant application could enhance vine plant production, but the positive effect depends on the climatological conditions and the plant location. Besides, the result obtained about WUE pointed to this physiological parameter as an excellent biomarker to predict vineyard yield.

Figure 4.9. *Health-status of BABA-treated grapes (A), distilled water-treated grapes (C) and Greetnal (GT)-treated grapes (C) from plot A at harvest (24 October 2018).*

The most studied parameters for defining the quality of the berries and must in vine plants treated with biostimulants are anthocyanins and phenolic compounds[161,407]. However, in this study, we focused on the content of sugars, free AAs, and AcOrg. It has been shown that the increase in temperatures in Europe, North America, and Australia affects the grape composition, inducing an increment in the sugar concentration and a decrease in acidity, ending with poor quality wines[413]. In this context, we observed that the application of biostimulants reduced the sugar content mainly in must and enhanced the accumulation of Fum. Wine freshness depends significantly on its acidity, and Fum has been reported to inhibit malolactic fermentation or stop it once initiated to preserve the wine's Mal content. ST-treated plants also accumulated Tart, Mal, and Acet together with Fum. Besides, this biostimulant contained higher levels of AcOrg between the three studied commercial substances (*Fig. 4.8* and *Table I.10* of **Annex I**). In wines, Mal, Tart, and Cit contribute the highest proportion to wine titratable acidity, whereas Tart can improve the color of red wines[414]. Altogether, applying *Ascophyllum nodosum*-based biostimulants can enhance the quality of the wines from areas affected by climate change. However, it is clear that the source of the raw material and the final product preparation condition the metabolic content of the biostimulants and, hence, their efficacy (*Fig. 4.10*).

Interestingly, BABA-treated plants also increased the BABA and Succ levels in musts. There is no information about the possible role of BABA in musts. It has been reported that at least the isoform GABA can serve as a source of nitrogen to produce Succ and that its assimilation improves yeast growth, fermentation rate, and glycerol production[415]. However, too much Succ induces a salty-bitter taste in the wine[416].

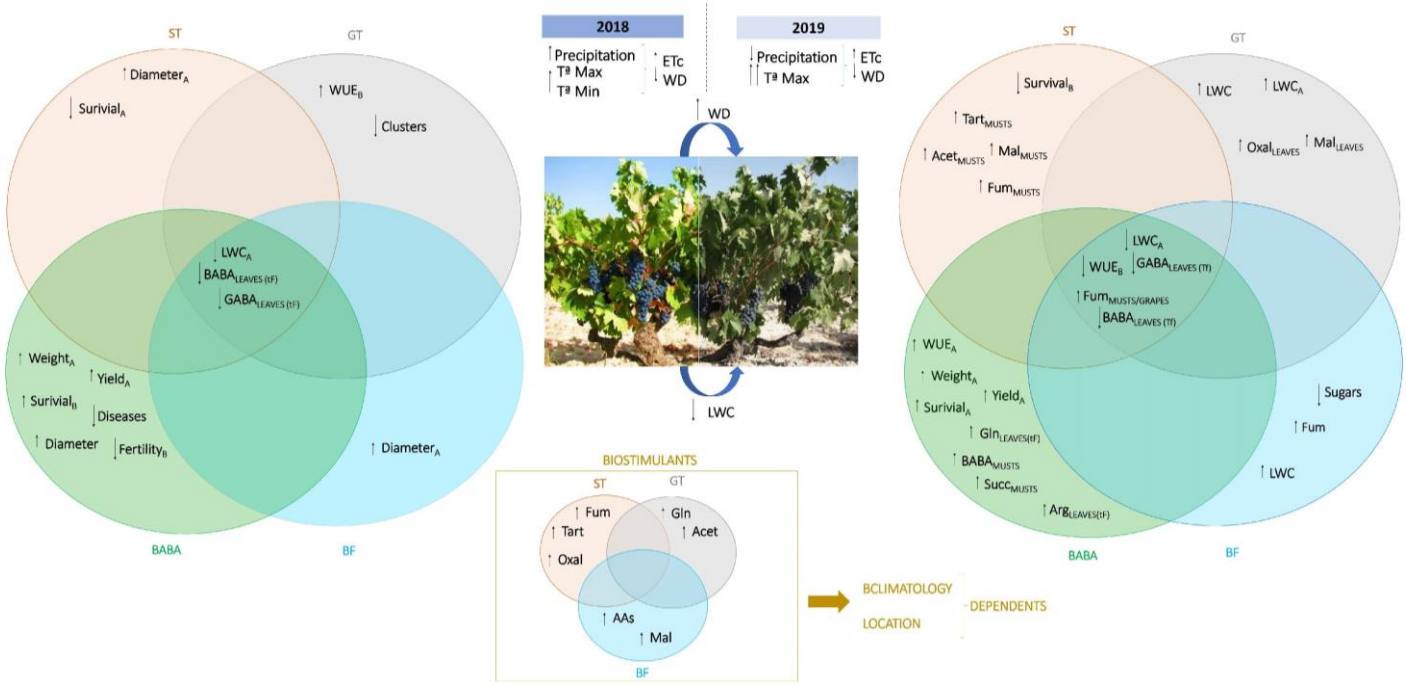


Figure 4.10. *Proposed mode of action in* Vitis vinifera *plants treated with the three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] in 2018 (moderate stress) or 2019 (severe stress).*

4.6 CONCLUSIONS

Biostimulants have been proven valuable for improving the wine's quality from vineyards located in areas highly affected by climatic change. They can enhance the content of AcOrg and reduce sugars, improving the quality of the final product (*Fig. 4.10*). However, not all biostimulants work the same way, including when the raw material source is the same. It is, then, essential to characterize them before their general use. Moreover, the application of biostimulants can also change the physiology and the final yield. However, in this case, the climatology and location of the vineyard also influence the plant response. Finally, we demonstrated that WUE could be a beneficial biomarker to predict the yield of the vineyard and hence the plant biostimulant efficiency.

Chapter V: Integrative Phenotyping of *Arabidopsis thaliana* for the biostimulants mode of action characterization

5.1 INTRODUCTION

Nowadays it is difficult to determine the mode/mechanism of action of many biostimulants on crops, since their activity is modulated by diverse factors that interact with each other such as the pant genotype, the growing conditions, dose, and application time[417]. For this reason, to ensure improved agriculture productivity, prevent pest resistance, and select stress-tolerant plants, it is necessary to understand the connection between observable plant characteristics (phenotype) and genetics (genotype)[418,419].

Plant phenotyping is considered the set of methodologies and protocols used to measure the characteristics of plants that can be expressed qualitatively or quantitatively from the cell level to the whole plants[420,421].

In recent years, several studies focused on developing non-destructive high-performance phenotyping (HTP) techniques to evaluate biostimulant activity in crops subjected to various stressors have been developed[422]. HTP approaches are mainly used in indoor phenotyping, consisting of non-invasive approaches based on sensors (cameras). Controlled plant phenotyping methods are recently becoming a very effective tool to characterize the mode of action of biostimulants of diverse nature and in many crop species[423-426]. These platforms can be classified according to the experiment or the scale, allowing the combination of several automated sensors[417,422]. These sensors aim to quantify photosynthesis, development, architecture, growth, or plant biomass production, traits that can accelerate plant breeding programs[423,427]. Integrative multisensory phenotyping is one of the methodologies used to describe the broad spectrum of plant traits, including visible images (with RGB cameras) and/or 3D images, spectroscopy images (hyperspectral images), infrared thermal imaging (NIR), and ChlF[365,422,424,428].

Water balance, gas exchange, and morphological parameters have traditionally been analyzed to determine plant drought tolerance[429-434]. In addition, the photosystem II (PSII) efficiency has been proved to play an essential role in water stress tolerance mechanisms[428,434]. Thus, the drought-induced changes in the plant physiology and morphology defined the plant's strategy to deal with the stress condition. For example, one of the most well-known tolerance strategies that the plants use to reduce water losses is the osmotic adjustment, which induces cell wall hardening and

promotes water entry due to the accumulation of active solutes[435]. Specific molecules such as soluble carbohydrates, free AAs, AcOrg, and free polyamines have been described as compatible solutes. Besides, they can also play an essential role as indicators of stress tolerance or as signal molecules[436-439].

Arabidopsis thaliana is an annual plant belonging to the Brassicaceae taxonomic family in the dicotyledonous group of vascular angiosperms plant[440]. Some Arabidopsis accessions have a short life cycle, between 6 and 8 weeks, and their genome, easily transmutable by flower immersion, is small, producing thousands of seeds in 4 months. For this reason, *Arabidopsis thaliana* is a plant model widely used as a universal reference species in plant biology[441,442]. Arabidopsis has also been extended to the plant phenotyping community, which mainly used this plant species for HTP screening approaches, including studying the mechanism of action of several biostimulants[417,442-444].

It has been described that plant biostimulants can influence the phenotypic traits of the plant, the stress tolerance, the absorption, and assimilation of nutrients, leaves pigmentation, photosynthetic efficiency, the number and area of leaves, the biomass of shoots and roots, the number of fruits and/or their weight, and hence improve the final yield[445-447]. However, the study of the biostimulant mechanism of action through phenotyping crops in natural field conditions presents complexities such as soil microflora, physical and chemical conditions of the soil, and climatic factors, making it difficult to reproduce. Non-invasive approaches allow a rapid and simultaneous evaluation of several morphological and physiological traits in a large number of plants under different growth conditions. However, in experiences carried out in the open field, there is greater complexity and variability, complicating the analysis and the data obtained[417,424,448]. The primary source of variability lies in that, plants grow in individual pots in the laboratory, while under open field conditions, plants compete with the entire canopy for water resources, and each plant can present a different genotype. For that reason, an individual evaluation of each phenotype is necessary[449]. Currently, phenotypic evaluation in *Vitis vinifera* has been primarily based on visual estimates or using the descriptors marked by the OIVV[450] or unique phenotyping platforms based on vehicles monitored with GPS[449]. However, studying this plant in the laboratory is not easy because of being a pluriannual and woody plant. It is then essential to evaluate the possible biological translation from an annual model plant like Arabidopsis to grapevines.

The HTP techniques using Arabidopsis under controlled conditions allow speeding up for characterizing biostimulants under concrete conditions (different stressors) or searching for new bioactive compounds and permitting the experiment's reproducibility[442-444]. Ugena *et al.* (2018)[404] and Sorrentino *et al.* (2021)[451] are good examples of the HTP protocols for characterizing the mechanism of action of different biostimulants, from complex compositions to small molecules based biostimulants. They demonstrated that the Multiple Trait High Throughput Screening (MTHTS) based on the semi-automatic analysis of Arabidopsis seedlings provides a powerful tool for the large-scale discovery of new potency biostimulants, including the characterization of its mode of action under optimal and stress conditions. The studies also included a straightforward Plant Biostimulant Characterization Index (PBC Index) defined through different morphological and physiological parameters to allow the classification of the biostimulants mechanism of action in three categories: i) growth or production promotors/inhibitors, ii) stress alleviators, and iii) combined action[404].

Finally, it is worth mentioning that as Brassicaceae, Arabidopsis is closely related to oilseed crops such as rapeseed, cauliflower, or radish, among others[440]. In this oleoproteaginous species, the seed oil and the proteins are determinants of economic value to the harvested seed[452,453]. Generally, seed oils are triacylglycerols (TAG), widely used for nutritional and industrial purposed and whose quality is determined by their fatty acid content[452,454,455]. Some authors studied the content and seed oil quality through Quantitative Traci Loci (QTL)[454,456-458]. Likewise, other studies revealed the role of fatty acid in plant morphology, growth, and development, and in response to stress[459-463]. Besides, their biosynthesis and modifications define the development of the seeds[464]. Altogether, we expect that biostimulants can influence the quantity and quality of the final yield in Arabidopsis, especially under stress conditions.

5.2 OBJECTIVES

The main aim of this study was to go further in the characterization of the commercial bistimulants tested in *Vitis vinifera* in chapter IV using MTHTS methods using Arabidopsis as a model plant.

The following partial goals were proposed to achieve this general objective:

- Evaluate different physiological parameters, such as water balance, gas exchange, chlorophyll fluorescence, or osmotic adjustment in Arabidopsis plants under water limitation treated with the three commercial biostimulants to determine tolerance indicators.
- Study the changes in the endogenous levels of different signaling molecules such as free AAs and AcOrg in response to drought and the recovery capacity of plants.
- Evaluate the final yield of Arabidopsis at the quantitative and qualitative levels by analyzing the fatty acids composition in the seeds.
- Define the mechanism of action for each commercial biostimulant studied under different water regimes.

5.3 MATERIALS AND METHODS

5.3.1 PLANT MATERIAL, GROWTH CONDITIONS, AND EXPERIMENTAL DESIGN

Seedling characteristics and growth conditions from *Arabidopsis thaliana* (accession Col-0) were performed as described in chapter III (section 3.2.1).

The phenotyping protocol took 50 days and consisted of several steps, including seed sowing, transferring seedlings into the soil, time-course RGB-imaging, ChlF-imaging, and IR-imaging. After the seedlings were transferred to the growth chamber under controlled conditions, the control and drought treatments were established into de PlantScreen™ Compact system. 27 days after, automated RGB-imaging, ChlF-imaging, and infrared-imaging were performed once a day every two days for the next 16 days as had been described in section 3.2.2.

5.3.2 WATER STATUS DETERMINATION

The rosette RWC (%) was determined three times during the exposure to limited water conditions. Measurements were made before the foliar application (27 DAS), after the first (32 DAS) and second (39 DAS) foliar biostimulants applications according to the *Eq. 3.8* described in section 3.2.4.

5.3.3 MORPHOLOGICAL PARAMETERS

Rosette area, perimeter, roundness, compactness, isotopy, eccentricity, RMS, and SOL was performed as described in section 3.2.5 of chapter III.

5.3.4 GAS EXCHANGE PARAMETERS

Gas exchange parameters and FTemp of Arabidopsis seedlings were determined in all plants as explained in chapter III (section 3.2.6). Besides, the leaf WUE was determined as the ratio between the instantaneous net photosynthesis and the foliar transpiration.

5.3.5 CHLOROPHYLL FLUORESCENCE IMAGING (ChlF)

ChlF measurements were acquired using an enhanced version of the FluorCam FC-800MF pulse amplitude modulated (PAM) chlorophyll fluorometer (Photon Systems Instruments, Czech Republic) in Arabidopsis seedlings untreated (C) or treated with the three commercial biostimulants grown with and without water limitation (24 days) as described in section 3.2.7.

5.3.6 BIOMETRIC AND GROWTH PARAMETERS

The fresh (FW, g) and dry (DW, g) weight of the Arabidopsis rosette and the aerial biomass expressed as the ratio between DW, and FW were determined twice, one day after the first (32 DAS) and the second (39 DAS) foliar application using *Eq. 3.2* described in section 3.2.8 of chapter III.

5.3.7 PRODUCTION PARAMETERS AND SEEDS OIL QUALITY

Yield-related parameters such as the Tot_number, Tot_weight, and 1,000_seeds were estimated in Arabidopsis seedlings for each treatment and growth conditions as described in section 3.2.9.1 of chapter III.

The fatty acids content of Arabidopsis seeds was measured by GC-MS as explained in section 3.2.9.2 of chapter III.

5.3.8 SOLUBLE OSMOLYTES QUANTIFICATION

Total AcOrg content (Oxal, Tart, Mal, Acet, Cit, Succ and Fum) was measured in 48 Arabidopsis seedlings at 32 DAS and 39 DAS according to the analytical methodology described in sections 3.2.10.1 of chapter III.

Finally, free AAs content AcOrn, AAA, BABA, GABA, Ala, β-Ala, Asn, Arg, Asp, Cis, Citr, Cys, Glu, Gln, Gly, His, Hpr, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Trp, Tyr, SAH and Val were quantified for 48 seedlings at 32 DAS and 39 DAS following the analytical methodology based on UPLC-MS/MS described in chapter III (Section 3.2.10.2).

5.3.9 PLANT BIOSTIMULANT CHARACTERISATION INDEX (PBC) FOR BIOSTIMULANTS MODE OF ACTION

The PBC Index was used as described by Ugena *et al.* (2018)[404] to characterize the three commercial biostimulants used in the study as growth or production promotors or inhibitors, stress alleviators, or a combined action with slight modifications.

The biostimulant characterization index has been only used to characterize plants at vegetative stages[404,451] based on morphological and physiological parameters. However, we proposed an extension of this index based on two PBC indices in this work. The first one integrates the significant physiological and morphological traits obtained from the multi-ANOVA and the second PBC index combines the quantity and quality yield-related parameters.

The relative changes between the number of the different analyzed traits from the treated Arabidopsis seedlings and the untreated plants were determined under well-watered and water limitation conditions. This number was used to calculate the log2 of the ratio. The values obtained were then summed up to calculate the PBC index, ending with a single numeric value that could straightforwardly categorize the compounds. The obtained number can be negative (red) or positive (blue), informing about the negative or positive effects on the plant performance of the treated plants compared to the respective control variant. This approach will help to classify the compounds as a plant growth promoter (only positive blue values in the treated seedlings under control conditions in PBC-growth index calculation), a production promoter (only positive blue values in the treated seedlings under control conditions in PBC-production index calculation), stress alleviators (positive values in seedlings under stress conditions) or both (positive blue values in seedlings under control and stress conditions). These numbers represented by the independent traits and treatment constituting the PBC index can be represented in a parallel coordinate plot for better understanding and visualization.

5.4 RESULTS

5.4.1 SOIL WATER CONTENT

Arabidopsis thaliana were weighed and watered from day 16 to day 40 DAS (*Fig. 5.1*. and Table 5.1). All the seedlings maintained optimal irrigation conditions until 17 DAS, and, progressively, two water regimes were induced; control conditions with 80% field capacity (FC) or water limitation defined as progressive drought (PD). The soil water content (%) for all treatments was estimated gravimetrically. All treatments showed a similar profile in soil water content under FC and PD. The first foliar spraying was carried out when the water soil capacity remained around 80.0±0.5% and 68.2±1.8% for FC and PD, respectively. The second foliar application was carried out under more stressful conditions when the water soil content for the Arabidopsis seedlings under PD was 52.4±2.1% (*Fig. 5.1B*). However, with deeper analysis, we observed some significant changes 24 h after the first biostimulant spraying (32 DAS). According to ANOVA, the spraying with GT and BF significantly increased the soil water content compared to C and ST plants under FC (*Fig. 5.1A*). However, when *Arabidopsis thaliana* seedlings grew under PD, no significant differences in soil water content were observed (*Fig. 5.1B*).

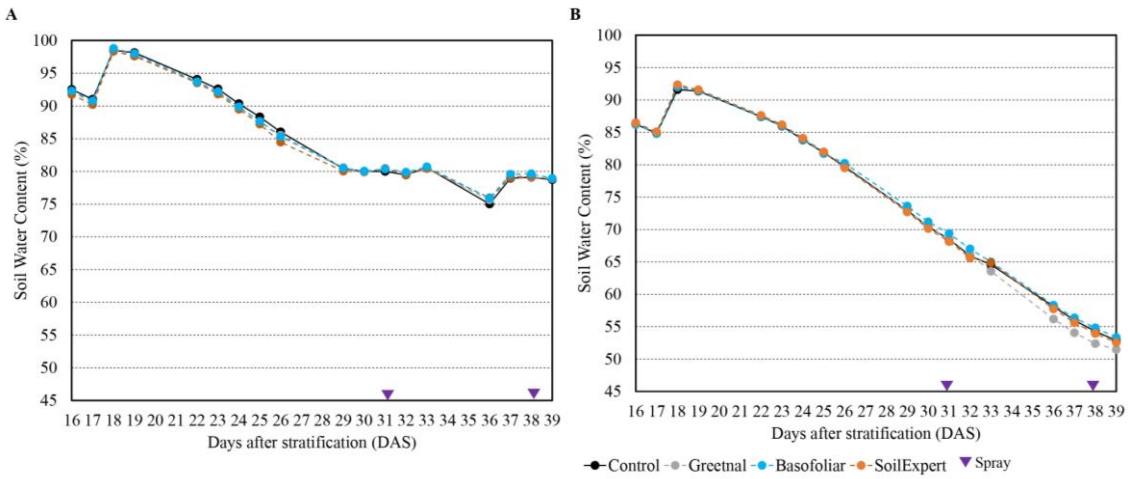


Figure 5.1. *Soil water content (%) (average) of Arabidopsis seedlings untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under A) field soil capacity (FC) or B) progressive drought (PD). Violet triangles indicated the moment of foliar application.*

Table 5.1. *Soil water content (%) [average ± standard error (SE)] of Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] subjected to field capacity (FC) and progressive drought (PD).*

DAS	FC				PD			
	C	GT	BF	ST	C	GT	BF	ST
16	92.5±1.1	92.1±1.0	92.3±1.4	91.7±1.2	86.3±1.2	86.2±1.3	86.2±0.8	86.5±0.9
17	91.0±1.1	90.6±1.0	90.8±1.4	90.2±1.2	84.8±1.2	84.9±1.3	84.8±0.8	85.1±0.9
18	98.5±1.0	98.6±1.1	98.8±1.0	98.3±1.2	91.6±1.1	92.2±1.1	92.0±0.6	92.3±1.0
19	98.2±0.9	97.8±0.9	98.0±1.0	97.6±1.0	91.4±0.9	91.5±1.0	91.4±0.7	91.6±0.9
22	94.1±1.0	93.5±1.0	93.7±1.0	93.6±1.0	87.4±1.0	87.5±1.0	87.4±0.8	87.6±1.0
23	92.6±1.0	92.0±1.0	92.2±1.1	91.8±1.0	86.0±1.0	86.1±1.1	86.0±0.8	86.2±1.0
24	90.3±1.0	89.8±1.0	89.9±1.0	89.5±1.1	83.8±1.0	83.9±1.1	84.0±0.9	84.1±1.1
25	88.3±1.1	87.6±1.0	87.6±1.1	87.2±1.1	81.9±0.9	81.8±1.1	81.7±0.9	82.0±1.1
26	86.0±1.1	85.5±1.1	85.3±1.2	84.5±1.0	79.7±0.7	79.5±1.2	80.2±0.7	79.5±1.2
29	80.4±1.0	80.6±0.5	80.5±0.7	80.0±0.3	72.9±0.9	72.9±1.6	73.6±1.2	72.7±1.7
30	80.0±0.2	79.9±0.2	80.0±0.2	80.0±0.2	70.4±1.0	70.4±1.7	71.2±1.4	70.2±1.8
31	80.0±0.5	80.5±0.1	80.4±0.2	80.4±0.4	68.5±1.0	68.5±1.7	69.4±1.5	68.2±1.8
32	79.5±0.2	79.9±0.2	79.8±0.1	79.5±0.3	65.9±1.1	66.2±1.7	67.0±1.7	65.6±1.9
33	80.6±0.3	80.5±0.3	80.7±0.5	80.4±0.1	64.6±2.0	63.6±3.0	65.0±1.6	64.9±2.7
36	75.0±0.6	75.7±1.0	75.9±0.8	76.0±0.6	58.1±2.3	56.2±2.4	58.3±2.1	57.8±2.5
37	79.0±0.3	79.1±0.6	79.6±0.1	79.1±0.5	56.0±23	54.1±2.1	56.4±2.0	55.6±2.3
38	79.1±0.2	79.2±0.7	79.6±0.7	79.3±0.4	54.3±2.1	52.4±1.9	54.8±2.0	54.0±2.1
39	78.7±0.4	78.9±0.5	78.9±0.3	79.0±0.3	52.9±1.8	51.5±1.2	53.3±1.9	52.6±1.8

5.4.2 MORPHOLOGICAL PARAMETERS

Visible RGB imaging was used to assess a range of visual traits in all plants under FC and PD (*Fig. 5.2*). Foliar application of biostimulants induced a fast change in the plant morphology. A visual elongation of the petioles was observed in the biostimulant-treated plants in ST and GT-treated plants under FC and PD compared to control plants. However, the BF-treated plants presented rosette damage and chlorosis at 45 DAS under both growth conditions, whereas ST and GT-treated plants showed a bigger size and were more open than C-untreated ones (*Fig. 5.2*).

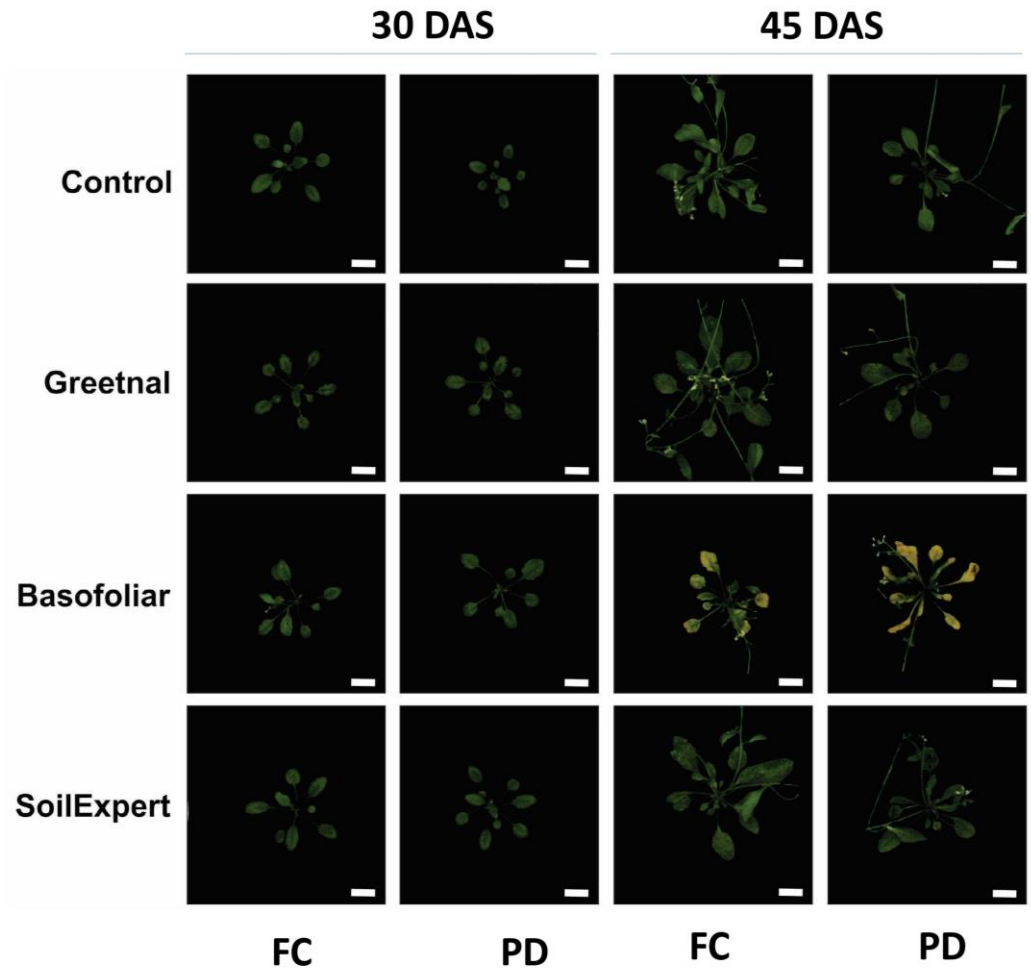


Figure 5.2. *Non-invasive RGB image of Arabidopsis seedlings untreated (Control) or treated with three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown under 80% field water capacity (FC) or progressive drought (PD) after the first foliar spray (30 DAS) and 15 days later (45 DAS).*

As the first step, we analyzed the Arabidopsis rosette area and perimeter changes from the RGB images (*Fig. 5.3*). A similar profile was observed in the growing curves of the plants under FC (*Fig. 5.3A and B*). Only BF-treated plants presented a reduced rosette area and perimeter at the end of the experiment. Under PD, an apparent beneficial effect on the growth was observed in the Arabidopsis seedling sprayed with ST and GT after the first foliar application (*Fig. 5.3C and D*). This tendency

was maintained until the end of the experiment. However, all plants reduced the area and perimeter two days after the second foliar application, especially in the ST and GT treatments.

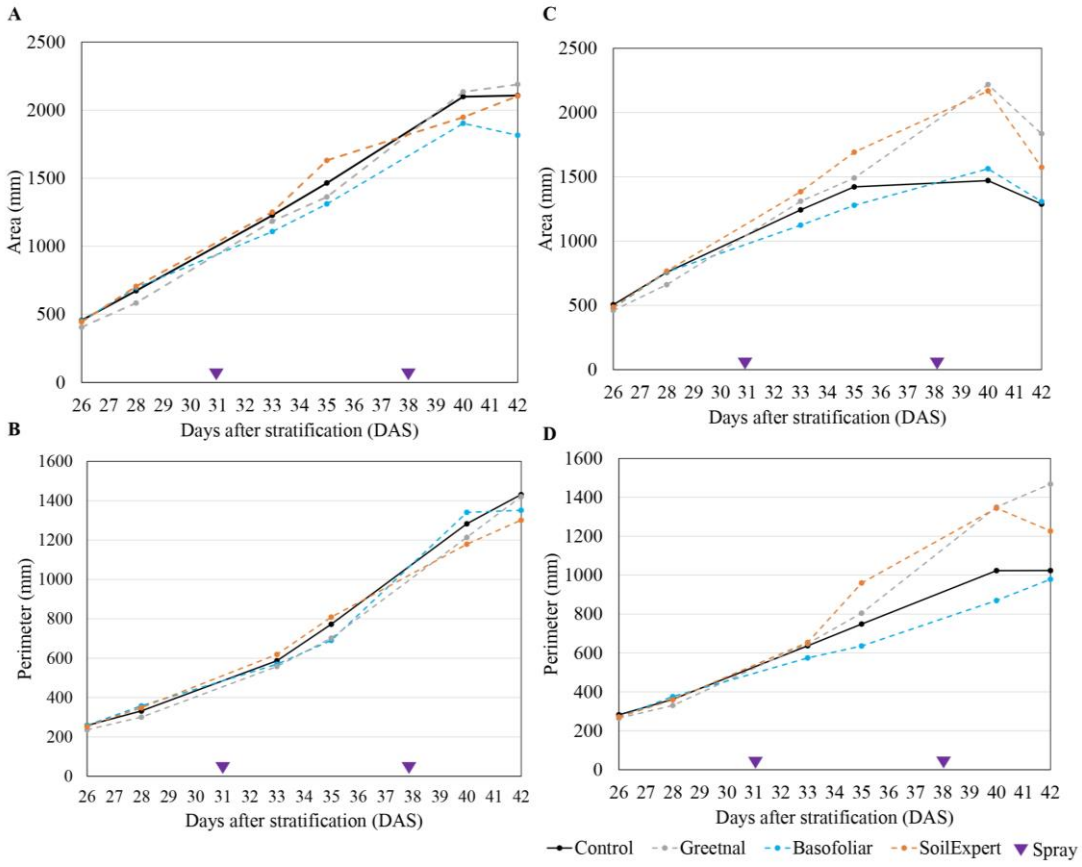


Figure 5.3. *Leaf area and perimeter (mm) [average ± standard error (SE)] in Arabidopsis seedlings untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field water capacity (FC) (A and B) or progressive drought (PD) (C and D). Violet triangles indicated the moment of foliar application.*

The main differences appeared after the foliar applications, but the differences between treatments depended on the growth conditions. For example, as observed in *Fig. 5.4*. C plants increased the slenderness of leaves and reduced the roundness 24 hours after the second spraying under PD *(Fig 5.4C and D).* Three days after, both the roundness and slenderness of the Arabidopsis rosette significantly increased in ST and GT-treated plants compared to control plants under PD. However,

all biostimulant treatments reduced these two parameters three days after the second foliar application under PD *(Fig 5.3C and D).*

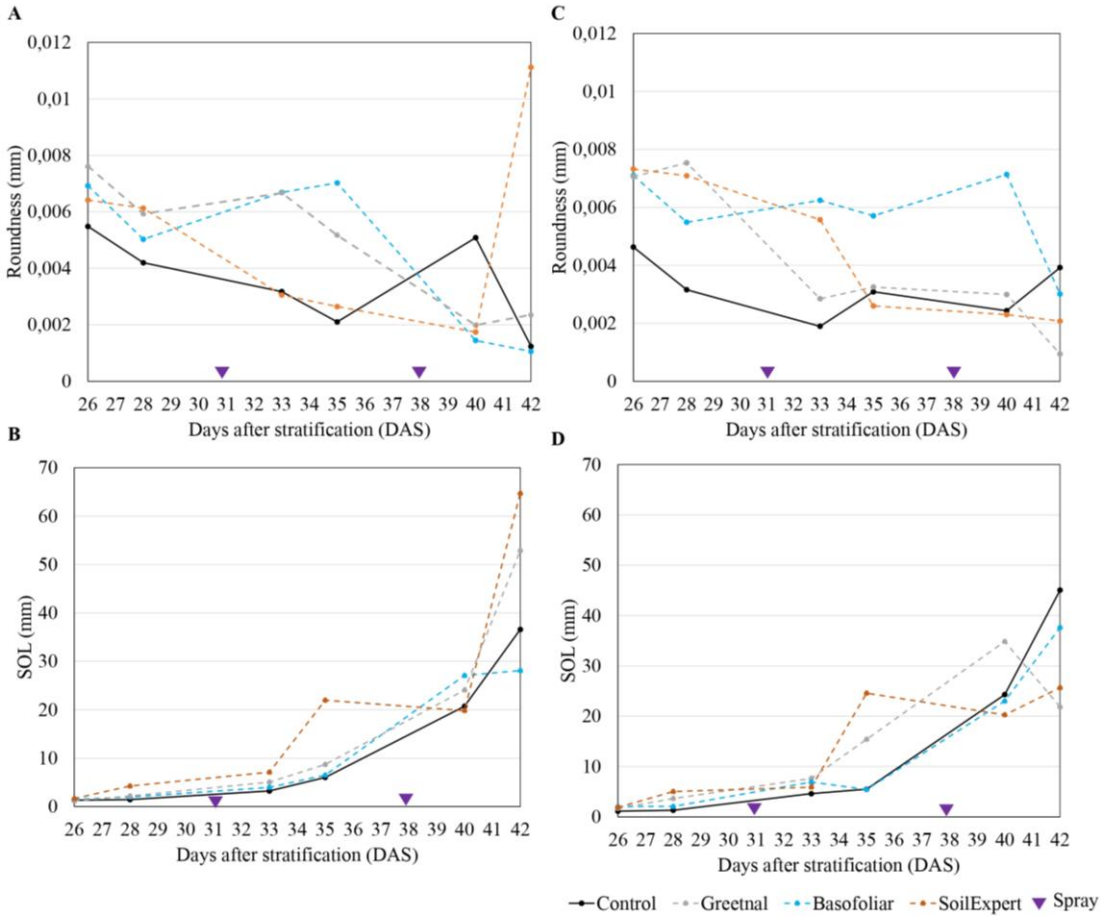


Figure 5.4. *Roundness and Slenderness [average ± standard error (SE)] of leaves (SOL) in Arabidopsis seedlings untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field water capacity (FC) (A and B) or progressive drought (PD) (C and D). Violet triangles indicated the moment of foliar application.*

The foliar application also affected the isotopy and compactness of the Arabidopsis rosette *(Fig. 5.5)*. In this case, BF was the biostimulant that increased both parameters most after the first foliar application (32 DAS) under FC and PD. However, BF-treated plants reduced the isotopy and compactness of the Arabidopsis rosette after the second foliar application under FC. In contrast, ST

increased them *(Fig. 5.5A and B)*. Under PD, the second spraying with BF maintained higher isotopy than control plants but removed the differences in compactness *(Fig. 5.5C and D)*.

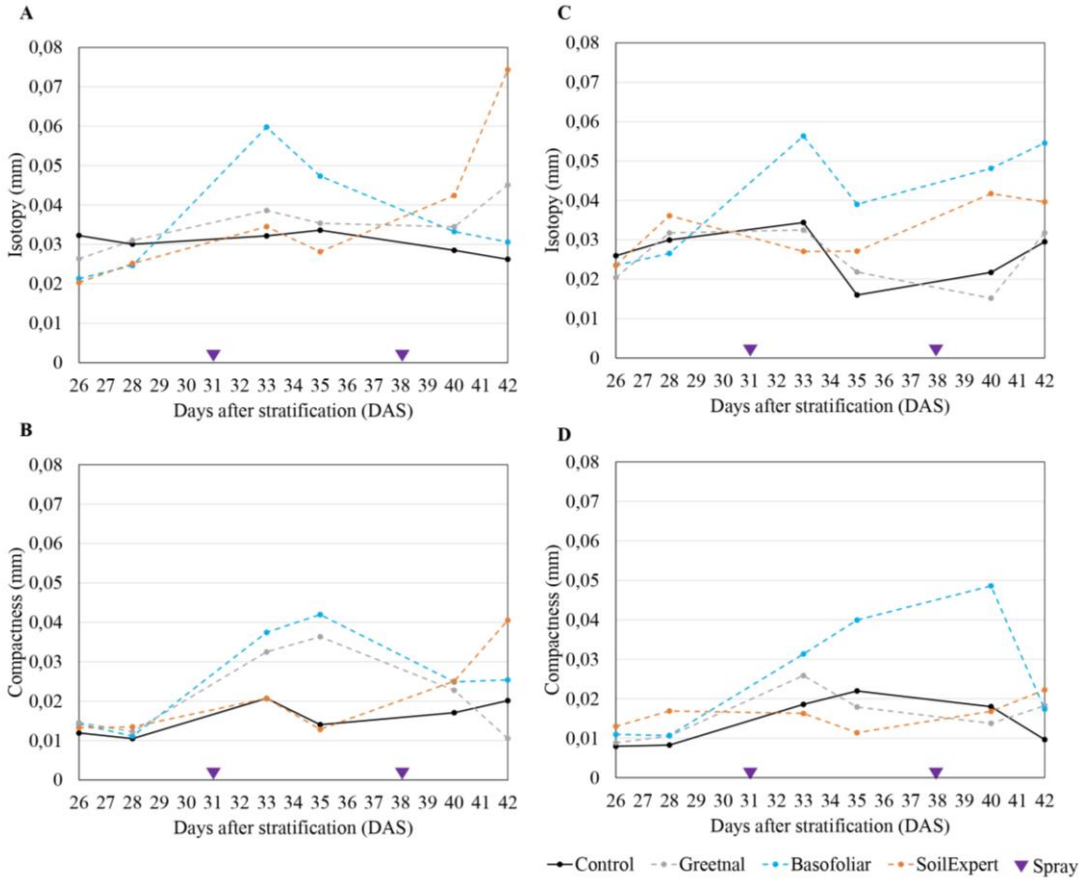


Figure 5.5. *Isotopy and compactness [average ± standard error (SE)] of Arabidopsis seedlings untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field water capacity (FC) (A and B) or progressive drought (PD) (C and D). Violet triangles indicated the moment of foliar application.*

Despite RMS and eccentricity did not present statistically significant differences, slight modifications were observed (*Fig. 5.6.*). Under FC, ST-treated plants presented higher RMS levels *(Fig. 5.6B*), whereas eccentricity tended to be higher in Arabidopsis seedlings treated with BF and GT under PD *(Fig. 5.6C)*.

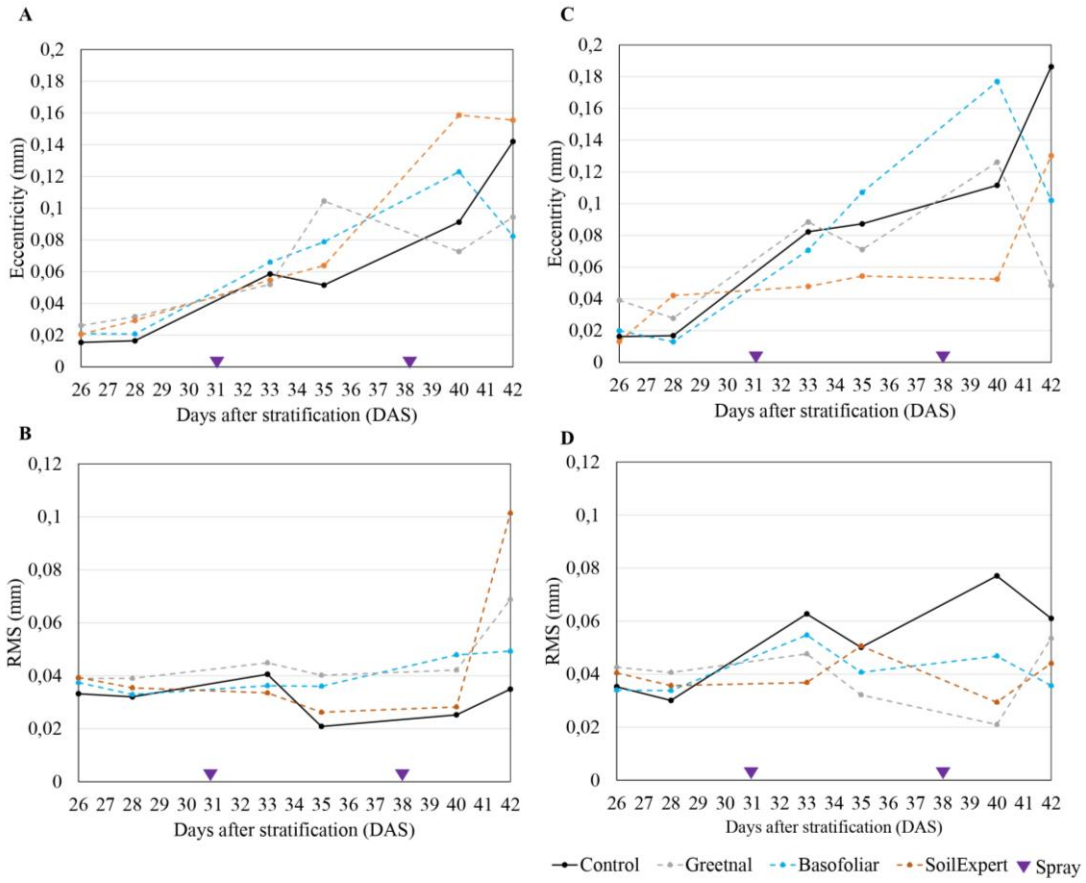


Figure 5.6. *Eccentricity and RMS [average ± standard error (SE)] of Arabidopsis seedlings untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field water capacity (FC) (A and B) or progressive drought (PD) (C and D). Violet triangles indicated the moment of foliar application.*

To go further in the analysis of the foliar application impact on the plants, the fresh weight (FW), dry weight (DW), and the biomass (DW/FW) were estimated manually at 32 DAS and 39 DAS (*Table 5.2)*. Foliar application of biostimulants affected plant biomass production. However, whereas BF and ST-treated plants increased the three parameters at both analyzed times compared to controls under FC conditions, GT-treated plants decreased them (*Table 5.2*). The favorable effect of the treatment was also observed in the ST and less extent in BF treated plants. Thus, the plants sprayed twice with ST accumulated 25% more FW and DW than the untreated ones under PD at 39 DAS, but this increase was not visible in the biomass, represented as DW/FW (*Table 5.2*). However, in BF-treated plants, the improvement was mainly in the DW, ending with better biomass

(10.5%) than controls (8.6%). Thus, we could conclude that the foliar application with biostimulants generally increases the weight of the Arabidopsis rosette under both FC and PD conditions.

Table 5.2. *Fresh weight (mg), dry weight (mg), and biomass (%) [(Average ± Standard error (SE)] in Arabidopsis seedlings in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under two growth conditions [Field capacity (FC, upper panel) or progressive drought (PD, lower panel)]. t32 and t39 indicate the time in days after sowing (DAS).*

FC

	FW_{t32} (mg)	**FW_{t39} (mg)**	**DW_{t32} (mg)**	**DW_{t39} (mg)**	**$Biomass_{t32}$ (%)**	**$Biomass_{t39}$ (%)**
C	107.5±29.5	467.5±65.0	9.4±2.2	41.1±5.3	9.2±0.9	8.8±0.3
GT	82.5±18.9	417.5±78.6	7.6±1.1	34.9±4.2	9.9±1.2	8.7±0.7
BF	115.0±21.8	530.0±45.5	11.6±2.6	51.2±3.9	9.9±0.6	9.7±0.3
ST	132.5±26.6	612.5±174.0	12.6±1.6	51.1±13.8	9.9±0.6	8.9±0.7

PD

	FW_{t32} (mg)	**FW_{t39} (mg)**	**DW_{t32} (mg)**	**DW_{t39} (mg)**	**$Biomass_{t32}$ (%)**	**$Biomass_{t39}$ (%)**
C	137.5±18.9	570.0±87.8	12.4±1.7	49.2±7.8	9.0±0.1	8.6±0.1
GT	135.0±15.5	537.5±135.8	12.1±1.6	45.5±12.1	8.9±0.4	8.3±0.2
BF	152.5±24.3	510.0±153.0	14.9±1.0	52.3±14.8	10.3±1.3	10.5±0.6
ST	120.0±4.1	710.0±85.0	10.6±0.4	61.4±6.8	8.8±0.3	8.7±0.1

5.4.3 PHYSIOLOGICAL PARAMETERS

As the next step, we performed a multi-ANOVA ($p<0.05$) to define the effect of biostimulants on *Arabidopsis thaliana* seedlings under the two opposite irrigation regimes (FC and PD) studied. The physiological parameters included gas exchange and chlorophyll fluorescence, WUE, and RWC. The biostimulant effect on Arabidopsis was evaluated separately by growth conditions (FC or PD) to define better their mechanism of action. The parameters that presented significant differences according to the multi-ANOVA are represented in *Table 5.3.*

***Table 5.3**. Statistical results based on multi-ANOVA for physiological parameters in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown under optimal irrigation conditions (FC, upper panel) or B) progressive drought (PD, lower panel).*

FC

	C	GT	BF	ST
C		A,gs,E,WUE		RWCt32,A,gs,E,WUE
GT				gs,E
BF	A,gs,Ci,E,WUE	A,gs,Ci,E,WUE		A,gs,Ci,E,WUE
ST				

PD

	C	GT	BF	ST
C		RWCt32,A,gs,E,QY_maxAUC		A,gs,Ci,E,WUE
GT				RWCt32, RWCt39,gs,Ci,QY_maxAUC,WUE
BF	A,gs,Ci,E,WUE	RWCt32, RWCt39,A,gs,E,QY_maxAUC		A,gs,E
ST				

*MultiANOVA assay present statistical significance(P<0.05) in; A: instantaneous net photosynthesis, *Ci: internal foliar CO_2 concentration in the suBFtomatic cavities, *gs: stomal conductance, *E: instant foliar transpiration, *WUE: instantaneous water efficiency, * RWC_{t32}: relative water content after first biostimulant application, * RWC_{t39}: relative water content after second biostimulant application, *QY_masAUC: Area under maximum PSII quantum yield curve.*

The relative water content (RWC, %) was measured at 27 DAS (RWC_{t27}), 32 DAS (RWC_{t32}), and 39 DAS (RWC_{t39}). The RWC of the Arabidopsis rosette was mainly affected by the biostimulant application PD (*Table 5.4*). The worst results were observed in the GT-treated plants that significantly decreased the RWC_{t32} and RWC_{t39} compared to control under PD. The application of BF and ST presented higher RWC_{t39} (62.6 and 63.8%, respectively) than control plants (56.7%), but the differences were not significative (*Table 5.3* and *5.4*).

Table 5.4. *Relative water content [(Average ± Standard error (SE)] in Arabidopsis seedlings before spraying (*RWC_{t27}*) and after first (*RWC_{t32}*) and second (*RWC_{t39}*) foliar applications in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under two growth conditions [Field capacity (FC) or progressive drought (PD)].*

		RWC_{t27}	RWC_{t32}	RWC_{t39}
FC	**C**	89.3 ± 3.8	86.8 ± 4.7	87.6 ± 4.6
	GT	88.4 ± 2.0	77.6 ± 1.9	85.8 ± 4.8
	BF	83.1 ± 3.6	77.3 ± 2.1	80.0 ± 1.0
	ST	91.1 ± 1.8	70.7 ± 5.9	76.9 ± 6.4
PD	**C**	91.5 ± 1.2	73.6 ± 3.4	56.7 ± 4.2
	GT	86.8 ± 6.4	50.5 ± 4.7	38.4 ± 6.6
	BF	86.4 ± 4.2	72.9 ± 3.4	62.6 ± 7.6
	ST	62.5 ± 2.2	63.8 ± 8.0	86.4 ± 3.2

Many fluorescence-related parameters were measured, including the biochemical quenching yield of the plants, as shown in *Fig. 5.7*. The untreated and treated plants did not show significant differences in some of the fluorescence-related parameters (*Table 5.3* and *Fig. 5.7*). The QY_max curve kinetics as the area under the curve (AUC) was reduced in the BF-treated plants compared to the other plants under FC conditions, mainly due to the changes after the foliar applications (*Fig. 5.7*). Under PD, the best-performed plants were those sprayed with GT that showed significantly higher QY_maxAUC than the other treatments, according to the multi-ANOVA (*Table 5.3* and *Fig. 5.7*).

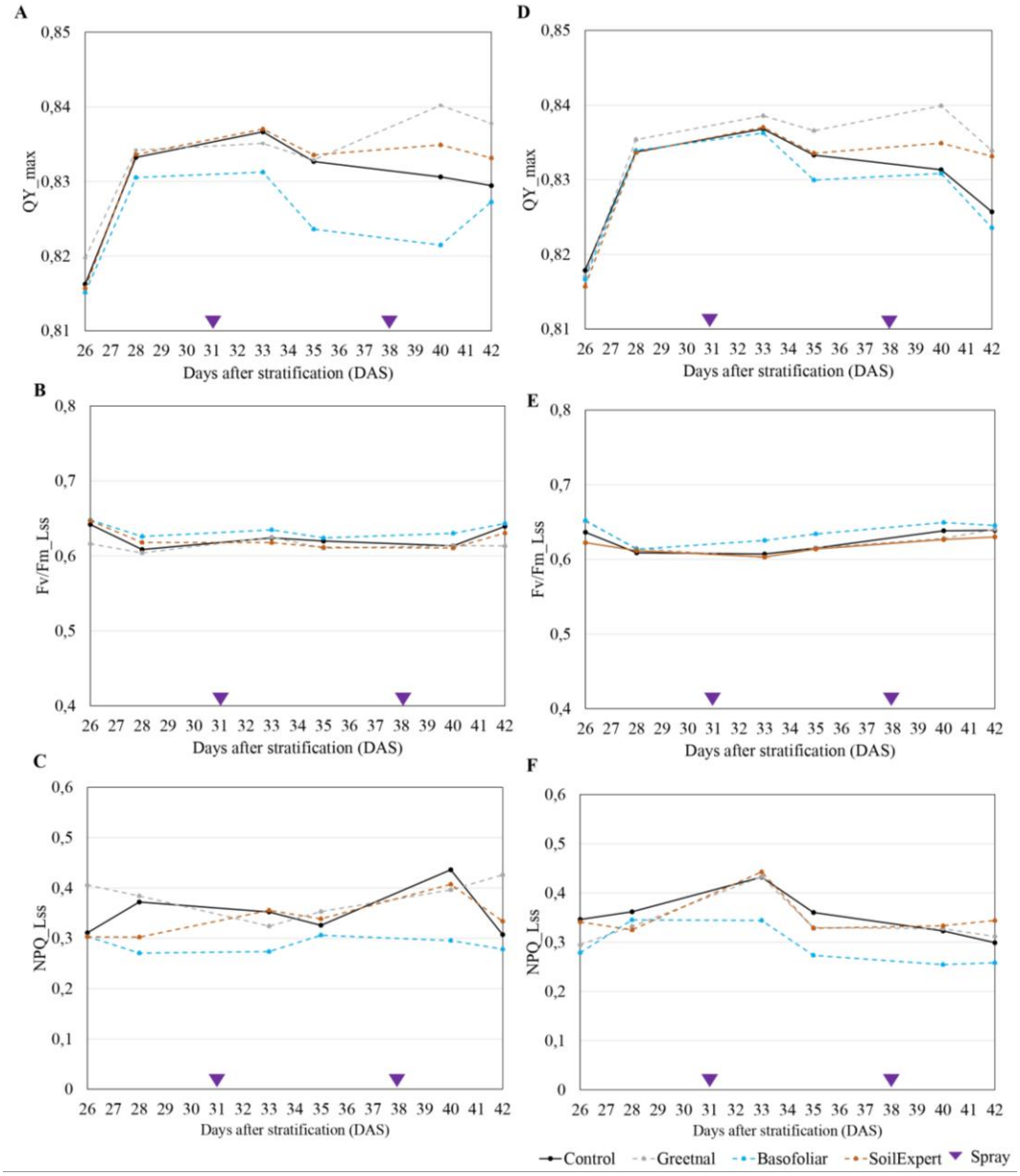


Figure 5.7. *Chlorophyll fluorescence-related parameters (Average) during the phenotyping protocol in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown under two growth conditions [Field capacity (FC) or progressive drought (PD)].; PSII quantum yield of the light-adapted sample at steady-state (Fv/Fm_Lss), maximum steady-state PSII quantum yield in light (QY_max). Steady-state non-photochemical quenching (NPQ_Lss) under field capacity conditions (FC) (A, B, and C) and progressive drought (PD) (D, E, and F). Violet triangles indicated the moment of foliar application.*

The foliar temperature was also measured using an IR camera. However, the differences were only due to the growth conditions but not the treatment effect. The Arabidopsis seedlings grown under FC conditions maintained the leaf temperature from 21.4 to 21.6°C along with the experiment, only a slight reduction was observed in the GT and ST-treated plants at the end of the experiment after the second application *(Fig. 5.8)*. Under PD conditions, the rosette temperature increased almost one degree from 21.4 to 22.2°C *(Fig. 5.8)*. Whereas the biostimulant-treated plants reduced the leaf temperature after the first foliar application, they increased it in the case of GT and ST after the second foliar application compared to controls and BF-treated plants.

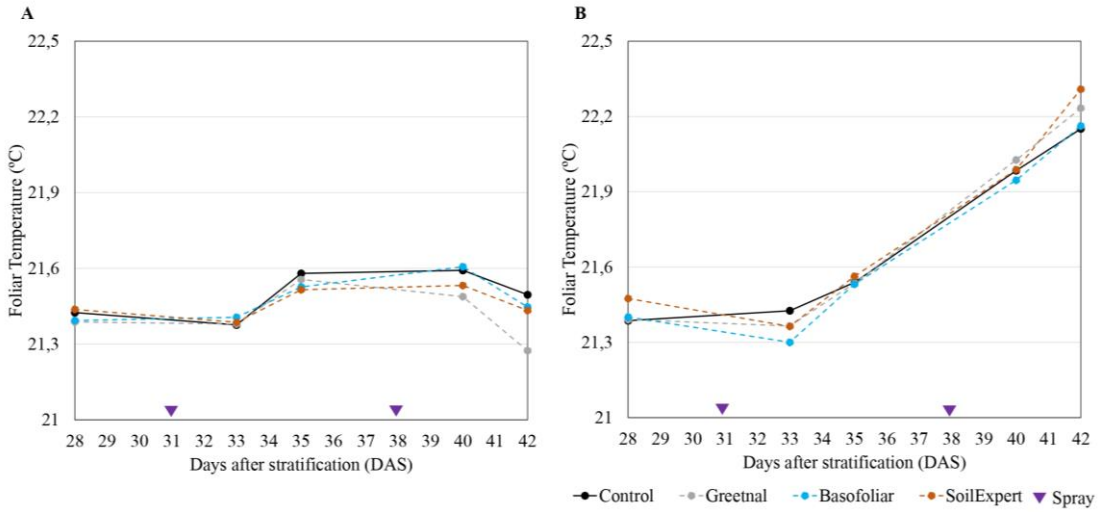


Figure 5.8. *Foliar Temperature (FTemp, ℃) of Arabidopsis seedlings untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown under A) field water capacity (FC) and B) progressive drought (PD). Violet triangles indicated the moment of foliar application.*

The control plants differed from the treated plants in almost all gas exchange parameters (A, gs, E) in both growth conditions. However, whereas GT and ST-treated plants increased the A and gs values compared to control under FC conditions, BF-treated plants reduced them (*Table 5.5*). Under PD, the foliar application with BF and ST also reduced the WUE compared to the control plants from 4.4±0.2 µmol CO_2 µmol^{-1} H_2O to 3.9±0.2 and 3.5±0.1 µmol CO_2 µmol^{-1} H_2O, respectively.

Table 5.5. *Gas Exchange parameters [instant foliar transpiration rate (E, mmol H_2O m^{-2} s^{-1}), stomal conductance to water vapor (g_s, mmol H_2O m^{-2} s^{-1}), instantaneous net photosynthesis assimilation rate (A, µmol CO_2 m^{-2} s^{-1}), and internal foliar CO_2 concentration in the subestomatial cavities (C_i, ppm CO_2)] and water use efficiency (WUE) [average ± standard error (SE)] in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] at 42 DAS grown under two growth conditions [Field capacity (FC) or progressive drought (PD)].*

		A	gs	Ci	E	WUE
FC	**C**	2.4 ± 0.1	92.8 ± 8.4	332.9 ± 3.8	1.3 ± 0.1	1.9 ± 0.2
	GT	2.9 ± 0.2	105.6 ± 9.5	335.5 ± 1.0	1.2 ± 0.1	2.3 ± 0.0
	BF	1.9 ± 0.1	59.8 ± 6.2	324.0 ± 5.3	0.7 ± 0.1	2.8 ± 0.2
	ST	2.8 ± 0.2	105.4 ± 6.5	336.8 ± 3.1	1.2 ± 0.1	2.4 ± 0.1
PD	**C**	2.0 ± 0.2	37.1 ± 4.2	284.5 ± 6.6	0.5 ± 0.1	4.4 ± 0.2
	GT	1.3 ± 0.2	21.9 ± 1.5	291.6 ± 6.6	0.3 ± 0.02	4.0 ± 0.3
	BF	1.5 ± 0.1	32.4 ± 3.1	299.9 ± 8.3	0.4 ± 0.04	3.8 ± 0.4
	ST	1.3 ± 0.2	22.9 ± 2.8	303.3 ± 9.2	0.3 ± 0.04	3.5 ± 0.2

To further study the mechanism of action of the biostimulants, we studied the changes of specific compatible solutes such as AcOrg and free AAs, whose accumulation can notably improve water maintenance, making the plant more tolerant of drought[361]. The accumulation of these metabolites has been studied at two different times; when drought conditions were considered moderate (after the first foliar application, 32 DAS) or severe (after the second foliar application, 39 DAS) stress conditions. At 32 DAS, the BF-treated plants significantly increased the total content of AAs (19070.7±2063.2 pmol g^{-1}) compared to controls (5252.0±589.5 pmol g^{-1}) under FC conditions (*Table 5.6*, *Table IX.2* of **Annex IX).** The main contributors to this increase were the metabolites Ile (52.1±10.6 pmol g^{-1}), Thr (1706.9±317.4 pmol g^{-1}), Ser (1303.4±40.3 pmol g^{-1}), Asn (1627.5±188.8 pmol g^{-1}), Asp (7449.6±927.8 pmol g^{-1}) and Lys (109.7±1.4 pmol g^{-1}).

The foliar application of GT treatment also induced a significant Lys (115.0±19.9 pmol g^{-1}) accumulation in the Arabidopsis seedlings compared to the untreated plants (*Table 5.6.* and *Table IX.2* of **Annex IX**).

At 32 DAS, when plants were under moderate drought, no changes in the metabolic content were observed among treatments compared to the control plants (*Table 5.6*). Only GT and BF application reduced the GABA content (15.8±3.0 and 25.3±2.1 pmol g^{-1}, respectively) compared to the control treatment (50.1±12.0 pmol g^{-1}).

Table 5.6. *Statistical results of multi-ANOVA for compatible solutes in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] at 32 DAS under two growth conditions [Field capacity (FC, upper panel) or progressive drought (PD, lower panel)].*

FC

	C	**GT**	**BF**	**ST**
C		Lys		
GT				Lys
BF	Ile,Thr,Ser,Asn,Asp,Lys,Tot_AAs	Asn,Asp		Ile,Val,Thr,Ser,Asp,His,Lys,Tot_AAs
ST				

PD

	C	**GT**	**BF**	**ST**
C				
GT				
BF		GABA		GABA
ST				

Ile: isoleucine, Thr: threonine, Ser: serine, Asn: asparagine, Asp: aspartic acid, Lys: lysine, Val: valine, His: histidine, Tot_AAs: total amino acids.

The metabolite content was also measured at 39 DAS, one day after the second foliar application (*Table 5.7*). As mentioned above, BF-treatment significantly increased the free AAs content compared to control under FC conditions (*Table 5.7, upper panel*). They showed significant accumulation of BABA (116.2±8.9 pmol g^{-1}), Ala (24.5±2.2 pmol g^{-1}), Thr (629.6±12.22 pmol g^{-1}), Gly (46.5±6.5 pmol g^{-1}), Ser (714.2±51.8 pmol g^{-1}), Asn (791.9±66.4 pmol g^{-1}), Cit (203.6±16.4 pmol g^{-1}), Asp (3463.1±315.0 pmol g^{-1}), HomoArg (197.4±10.2 pmol g^{-1}), His (24.2±2.3 pmol g^{-1}), Lys (371.1±89.7 pmol g^{-1}) and total acetylamino acids (Tot_AcAAs). However, ST and GT-treated plants only accumulated Asn (543.5±58.9 pmol g^{-1} and 486.6±23.4 pmol g^{-1}, respectively), Asp (548.0±65.4 and 1645.2±216.5 pmol g^{-1}) and SAH (1.1±0.4 and 1.4±0.3 pmol g^{-1}) compared to control.

Under PD conditions, the foliar application of the three commercial biostimulants increased the total amino acid accumulation (4722.7±334.5 pmol g^{-1} for GT, 10398.9±1148.5 pmol g^{-1} for BF, and 5903.8±585.5 pmol g^{-1} for ST) compared to control treatment (1492.0±365.6 pmol g^{-1}) (*Table 5.7, lower panel,* and *Table IX.3* of **Annex IX**). All of them increased the content of Thr, and GT and ST also increased Cit, among others. Altogether, it was clear that the foliar application of biostimulants increases the content of certain free AAs in Arabidopsis plants under FC and PD conditions. However, the type of metabolite and the amount varied with the number of applications, type of biostimulant, and growth conditions in Arabidopsis. In this regard, similar behavior was observed between GT- and ST-treated plants, maybe due to the exact nature of their raw material.

Table 5.7. *Statistical results of multi-ANOVA for compatible solutes in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] at 39 DAS under two growth conditions [Field capacity (FC, upper panel) or progressive drought (PD, lower panel)].*

FC

	C	**GT**	**BF**	**ST**
C		Thr,Asn,Cit,Tot_AAs		Cys,Thr,Cit,Asp,Tot_AAs
GT				Cys,Asp
BF	Ala,Thr,Glu,Ser,Asp,Arg,Lys,Tot_AAs	Asp,Lys		Cys,Glu,Lys
ST				

PD

	C	**GT**	**BF**	**ST**
C		Asn,Asp, SAH		Asn
GT				
BF	BABA,Ala,Thr,Gly,Ser,Asn,Cit,Asp,Homo Arg,His,Lys,SAH, Tot_AAs,Tot_AcAAs	Gly,Glu		Cit,Asp
ST				

BABA: β-aminobutyric acid, Ala: alanine, Thr: threonine, Glu: glutamic acid, Ser: serine, Asn: asparagine, Cit: citrulline, Gly: glycine, Arg: arginine, Asp: aspartic acid, Cys: cystine, HomoArg: homoarginine, His: histidine, Lys: lysine, SAH: S-Adeninhomocisteine, Tot_AAs: total amino acids, Tot_AcAAs: total acetyl-amino acids.

5.4.3.1 MULTIVARIATE STATISTICAL ANALYSIS OF THE PHYSIOLOGICAL AND BIOCHEMICAL CHANGES

To better understand the biostimulant mechanism of action, different multivariate statistical analyses were performed, in which several principal component analyses (PCA) and correlation matrices were performed. For the analysis, the data shown in *Table IX.1 to Table IX.4 of* **Annex IX** has been processed, centering them on their average values. First, Pearson´s correlation analysis between physiological parameters and metabolites from all plants grown under moderate stress conditions (32 DAS) was performed and represented in a matrix *(Fig. 5.9.*). Several correlations between them were observed. For example, a higher RWC was positively correlated with the Fum content and negatively correlated with many free AAs such as *β*-Ala, AcOrn, His, Leu, Ile, Asp, Thr, Ser, Homo Arg, Ala, AcGlu, Hpr, SAH, Gln, Pro, Tyr, Trp, Val, and Tot_AcAAs *(Fig. 5.9.*). Besides, the FW and DW of the plants were positively related to the Gln content and negatively to Gly, AcOrn, Orn, Glu, and Cit.

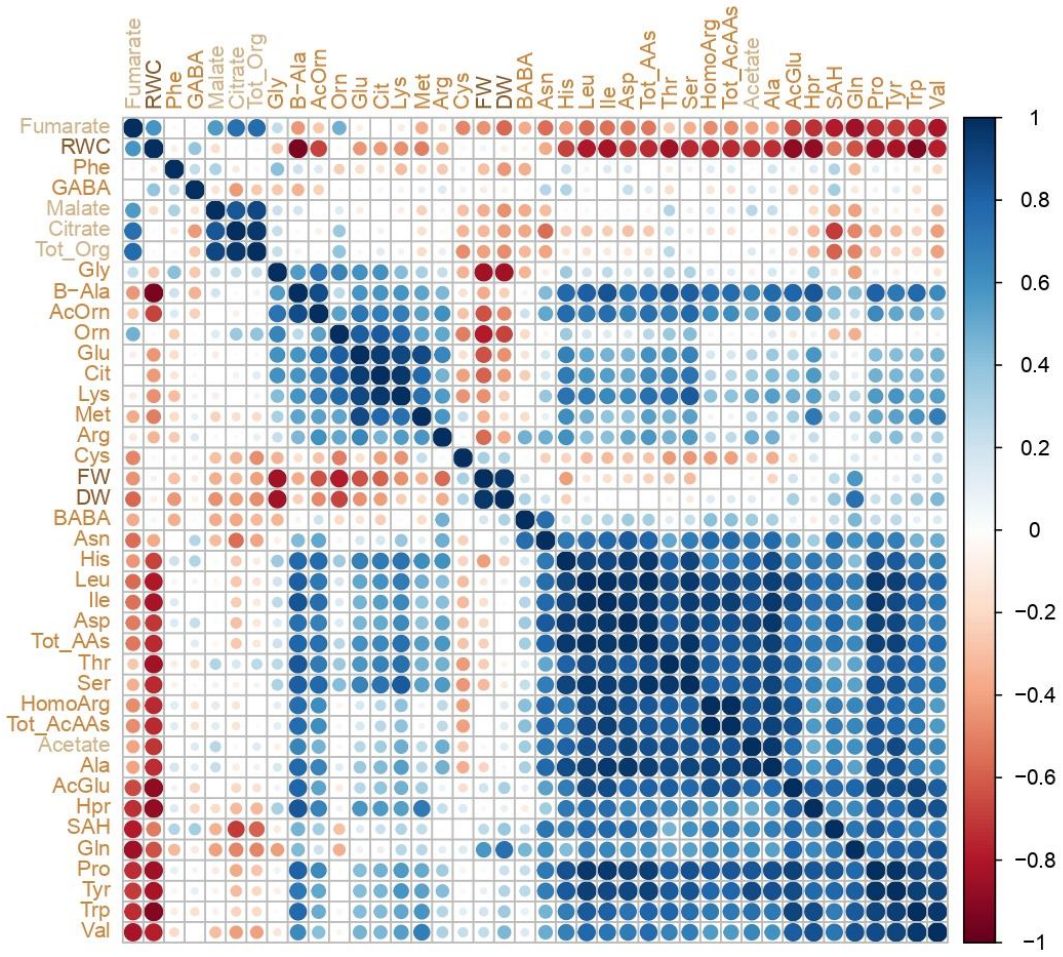


Figure 5.9. *Pearson´s Correlation analysis under moderate drought (32 DAS).*

In the second step, we performed two PCAs; to study the effect of the biostimulant application under FC *(Fig. 5.10)* or PD *(Fig. 5.11)* conditions. The PC1 and PC2 explained a total model variance of 90.25% for FC and 79.81% for PD. *Fig. 5.10* presented a new grouping between ST and Control treatments due to their gain in fresh weight (FW) and AAs accumulation such as GABA, BABA, and Cys. In contrast, GT was related to the higher total content of organic acids (Tot_Org), especially Cit, Fum, Mal, and free AAs such as Orn and Cit. However, BF was grouped with Tot_AcAAs, Tot_AAs, the Acet, and many free AAs, including Leu, Ile, AcGlu, Trp, Val, Ala, Asp, Pro, Tyr, HomoArg, Phe, Glu, SAH and Asn.

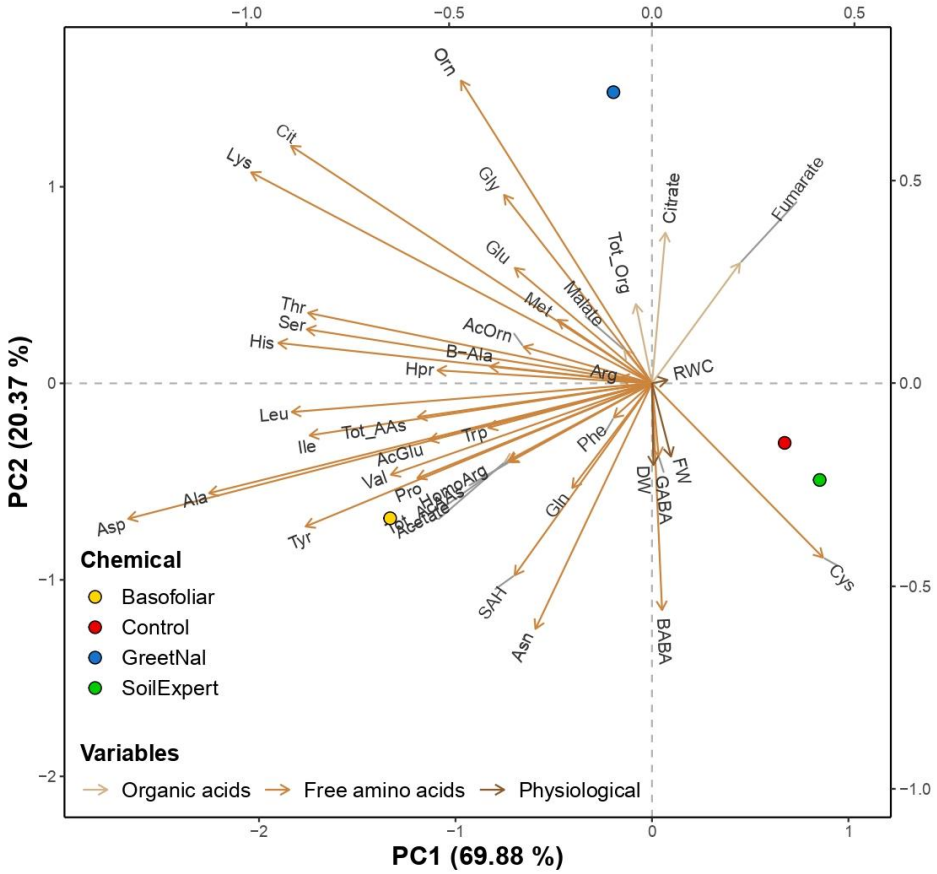


Figure 5.10. *Principal component analysis (PCA) based on physiological parameters and metabolites in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] at 32 DAS under field capacity (FC) conditions.*

Under moderate stress conditions (*Fig. 5.11*), the behavior of ST and GT was grouped closer and related to higher Tot_AcAAs, HomoArg, Thr, and the Acet and Cit. They were also opposed to the controls that presented higher GABA and RWC. Finally, BF-treated plants were grouped mainly by their total content of Tot_AAs and AAs like Pro, Val, Met, and Glu, and less Fum and Phe. Altogether, it is clear that the foliar application with biostimulants modifies the metabolism when the plants are grown under optimal or moderate water stress conditions.

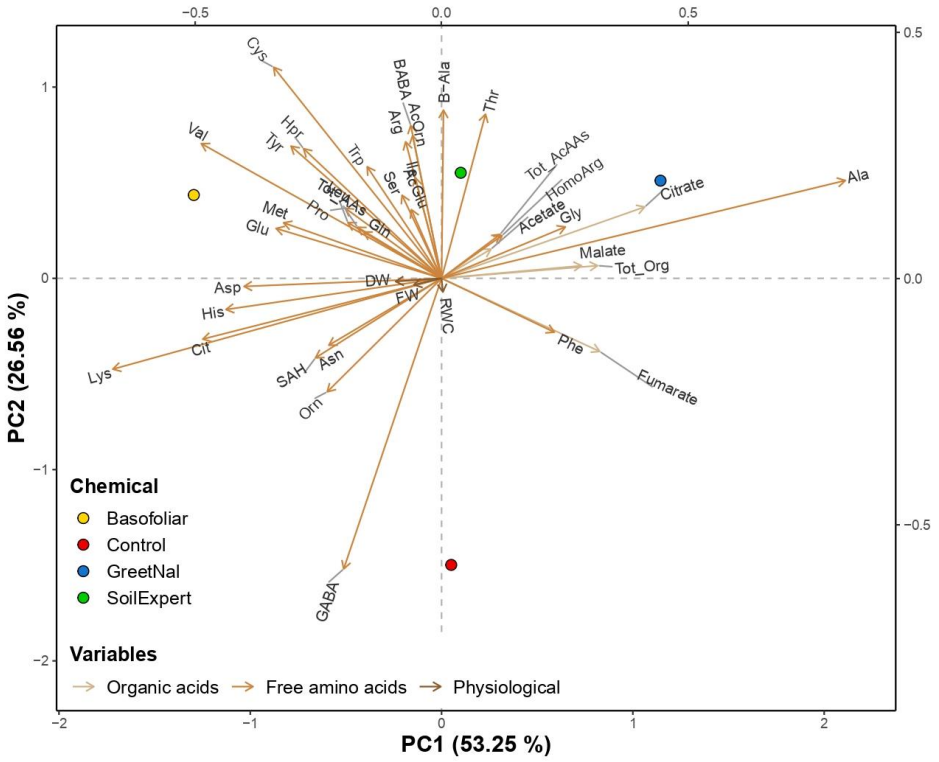


Figure 5.11. *Principal component analysis (PCA) based on physiological parameters and metabolites in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] at 32 DAS under progressive drought (PD) conditions (moderate drought stress).*

Finally, a completed study of the most significant physiological parameters was done under severe stress conditions. Similarly, a correlation matrix showed Pearson´s correlations between parameters and metabolites (*Fig. 5.12*). At this point, data from the gas exchange and chlorophyll fluorescence-related parameters were also included. A positive effect between gas exchange parameters and RWC of the Arabidopsis rosettes was observed. However, they presented an inverse correlation with the foliar temperature, steady-state PSII quantum yield, steady-state PSII quantum yield in light (QY_Lss), and PSII quantum yield of the light-adapted sample at steady-state PSII quantum yield in light-adapted steady-state (Fv/Fm_Lss), and higher energy dissipation (NPQ_Lss), with the biomass-related parameters FW and DW, with a WUE, and the AAs as AcOrn and Gln. In addition, higher values on the fluorescence-related parameters were inversely correlated to the accumulation of Ser, Arg, AcOrn, Val, Ile, Orn, Tyr, Lys, Leu, Phe, Gly, Hpr, BABA, Pro, Tot_AAs, and the Acet

and Mal. Finally, the area under the curve of the RGB data (RGB-AUC, kinetics of the green pixels) positively correlated to the levels of AcGlu accumulation and Met and reduced the vitality of the seedlings (Rdf_Lss) (*Fig. 5.12*).

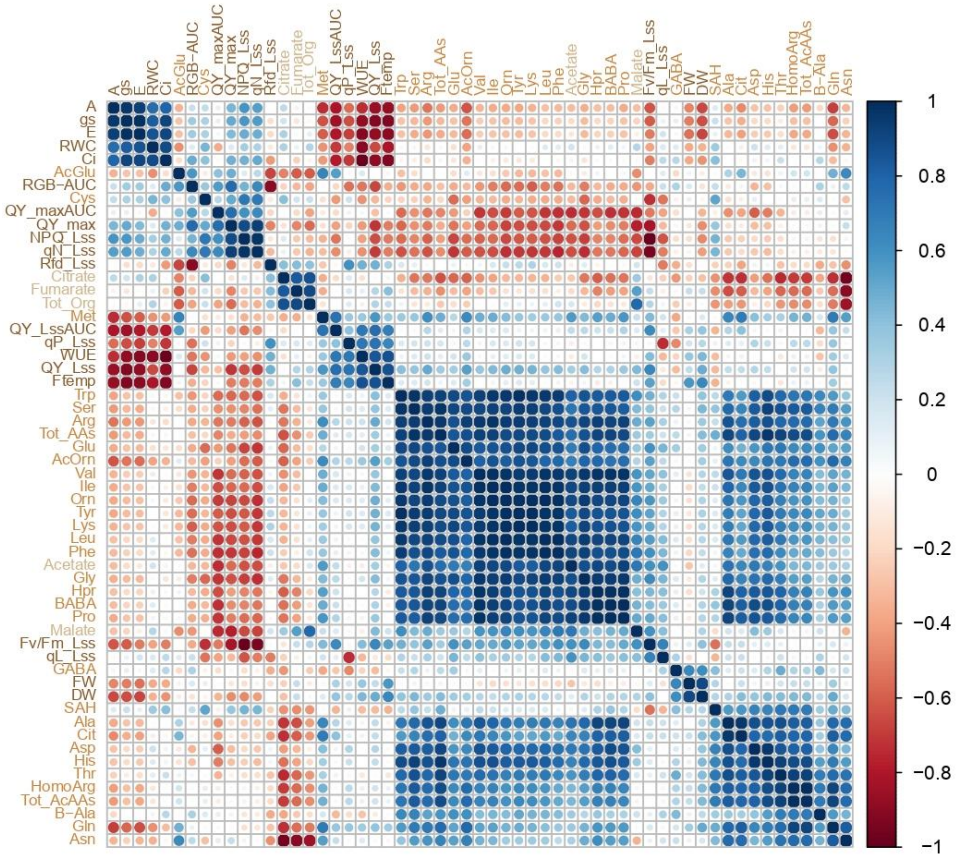


Figure 5.12. *Pearson´s Correlation under severe stress conditions (39 DAS).*

At 39 DAS, two additional PCAs were performed to further the biostimulant characterization. The variance of the experimental model accumulated at 93.77% and 90.99% for FC and PD conditions, respectively *(Fig. 5.13* and *5.14)*. Under FC (*Fig. 5.13*), control plants were more influenced by parameters such as RWC, photosynthetic parameters like PSII quantum yield of the light-adapted leaf at steady-state (Fv/Fm_Lss), Tot_Org, and the content of organic acids as Fum and Cit *(Fig. 5.13)*. They were also inversely correlated to Tot_AcAAs, HomoArg, Asn, Ala, and SAH. Finally, BF-treated plants were grouped with many AAs, including AcOrn, Hpr, Glu, Trp, Gly, Arg, Val,

Pro, Ile, Tyr, Leu, Met, Phe, and the organic acids Acet. The control plants were opposite to the ST and GT-treated plants. These treated plants were closely located and showed a positive correlation to the chlorophyll fluorescence related parameters (See *Fig. 5.7* and *Fig. IX.1* of **Annex IX**), such as their maximum PSII quantum yield (QY_max), fluorescence decline ratio in steady-state (Rfd_Lss), coefficient of non-photochemical quenching in steady-state (qN_Lss), steady-state non-photochemical quenching (NPQ_Lss) and the gas exchange parameters such as instantaneous net photosynthesis assimilation rate (A) and stomatal conductance (gs). Likewise, these treatments also improved the accumulation of the AAs β-Ala and reduced Acet and Mal, and the free AAs Orn, Phe, and Pro, among others *(Fig. 5.13)*.

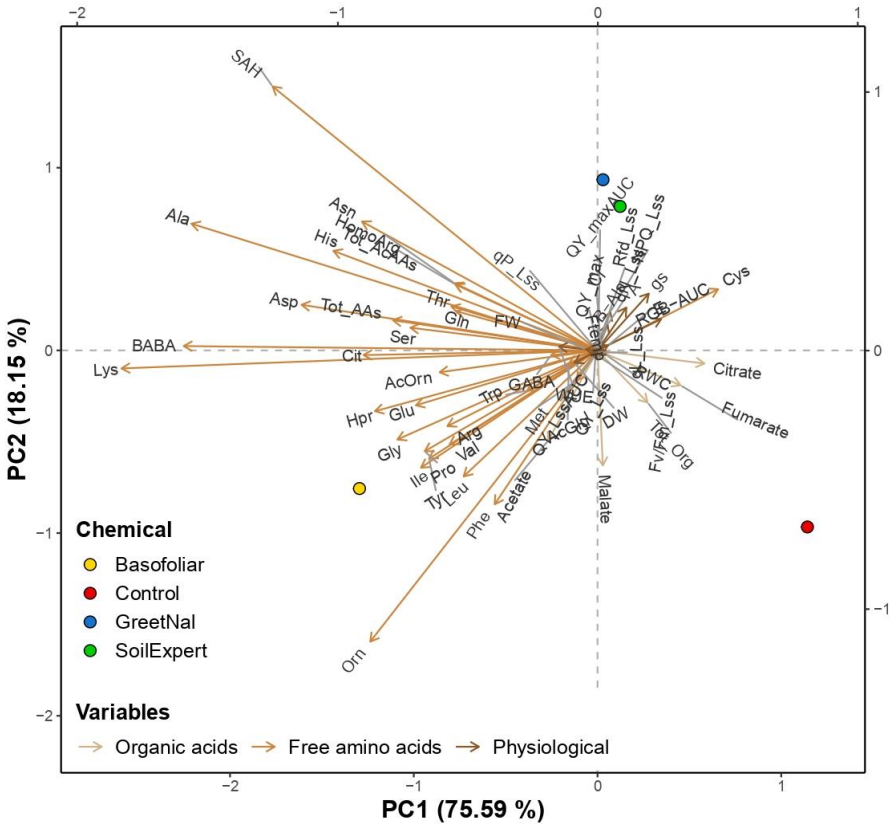


Figure 5.13. *Principal component analysis (PCA) based on physiological parameters and metabolites in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] at 39 DAS under field capacity conditions (FC).*

When the plants under severe drought conditions were analyzed (*Fig. 5.14.*), the PCA obtained grouped the plants similarly as observed under moderate drought stress (*Fig. 5.11*). Again, ST and GT-treated plants were closely located. They showed a positive correlation with chlorophyll fluorescence parameters such as the steady-state PSII quantum yield in light (QY_Lss), steady-state non-photochemical quenching (NPQ_Lss), and maximum PSII quantum yield (QY_max), and biomass related parameters such as RGB_AUC and the fresh rosette weight (FW). Finally, BF-treated plants were correlated to higher levels of free AAs such as Gly, Phe, Glu, Leu, Val, Pro, Trp, Ile, Tyr, Orn and Lys, the organic acid Acet. It is worth mentioning that BF-treated plants accumulated doubled Acet than the rest of the treatments and controls (**Annex IX**, *Table IX.4*).

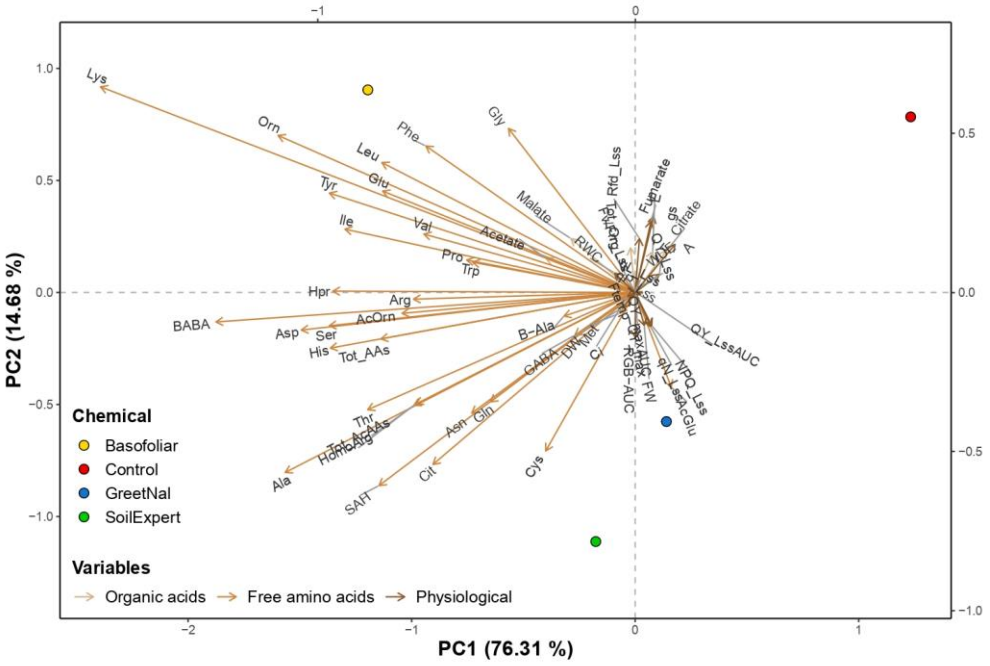


Figure 5.14. *Principal component analysis (PCA) based on physiological parameters and metabolites in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] at 39 DAS under progressive drought conditions (severe drought).*

The results showed that the foliar application of biostimulants affects Arabidopsis morphology, physiology, and metabolism. However, the mechanism of action of each biostimulant is different and highly influenced by its composition. For example, BF is a formulation rich in AAs, so its foliar

application induces the accumulation of several AAs in the plant, independently of the growth conditions. However, it also increased the accumulation of the organic acid Acet. Although they are considered compatible solutes, the free AAs accumulation does not always ensure a better water balance, as observed in moderate stress.

ST and GT formulation is prepared from extracts of *Ascophyllum Nodosum* from two companies. However, their foliar application improved the photosynthetic parameters of the plants under FC and PD conditions. It was especially remarkable under severe drought conditions, in which the treated plants tended to have better growth (RGB-AUC and FW), higher values of chlorophyll fluorescence-related parameters, and accumulated more AcGlu.

5.4.4 PRODUCTION AND QUALITY PARAMETERS

As the final step to evaluate the effect of the biostimulant application in Arabidopsis plants, six plants per treatment (control or treated with BF, GT, or ST) and growth conditions (FC or PD) were kept for production (*Table 5.8*), and the data analyzed using multi-ANOVA (*Table 5.9*). The results showed that the differences in the plant yield were more due to the growth condition but not to the treatment. The differences were mainly due to the changes in the Tot_number of seeds and the Totl_weight of seeds produced per plant. Whereas the control plants showed a Tot_weight of seeds per plant of 95±6 mg and a Tot_number of seeds per plant of 4972±578 under FC conditions, they reduced to 39±5 mg and 1799±282 under PD (*Table 5.8*). When the data were analyzed by growth conditions, an evident increase in the Tot_number of seeds per plant in the biostimulant treated Arabidopsis seedlings under FC conditions (*Table 5.8, upper panel*). Thus, GT and ST-treated plants increased the Tot_number of seeds per plant by over 9% from the controls, whereas BF-treated plants produced 5.9% more (*Table 5.10)*. Moreover, GT-treated plants also increased a 12.6% more the Tot_weight of seeds per plant. However, the weight of 1,000 seeds was the same for all treatments (20 mg), except for ST-treated plants with 18 mg (*Table 5.8, upper panel).*

Table 5.8. *Production parameters [Average ± Standard error (SE)] in Arabidopsis plants untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field capacity conditions (FC) and progressive drought (PD).*

FC

	Tot_weight (mg)	1,000_seeds	Tot_number
C	95 ± 6	20 ± 2	4972 ± 578
GT	107 ± 8	20 ± 1	5446 ± 244
BF	100 ± 8	20 ± 1	5263 ± 688
ST	98 ± 6	18 ± 1	5434 ± 513

PD

	Tot_weight (mg)	1,000_seeds	Tot_number
C	39 ± 5	22 ± 1	1799 ± 282
GT	24 ± 2	20 ± 1	1221 ± 105
BF	29 ± 5	19 ± 2	1527 ± 250
ST	28 ± 2	24 ± 3	1332 ± 251

Table 5.9. *Statistical results of multi-ANOVA assay obtained for the biostimulant treatment under optimal irrigation conditions (FC) and progressive drought (PD) based on production parameters.*

	C (PD)	**C (PD)**	**GT (FC)**	**GT (PD)**	**BF (FC)**	**BF (PD)**	**ST (FC)**	**ST (PD)**
C (PD)					Tot_weight, Tot_number			
C (FC)		Tot_weight, Tot_number				Tot_weight, Tot_number		
GT (PD)	Tot_weight, Tot_number				Tot_weight, Tot_number			
GT (FC)		Tot_weight, Tot_number		Tot_weight, Tot_number		Tot_weight, Tot_number		
BF (PD)						Tot_weight, Tot_number		
ST (PD)								
ST (FC)	Tot_weight, Tot_number	Tot_weight, Tot_number	Tot_weight, Tot_number	Tot_weight, Tot_number	Tot_weight, Tot_number	Tot_weight, Tot_number		Tot_weight, Tot_number

Under PD conditions (*Table 5.8, lower panel)*, the foliar application with biostimulants showed rather adverse effects on the Arabidopsis yield, so all treated plants reduced the Tot_weight of seeds per plant and the Tot_number of seeds per plant compared to control. ST treatment was the less effective, reducing the weight of the seeds per plant to 46.1% from the control values. However, the ST application improved the weight of 1,000 seeds by 9.1% compared to the control at the same growth conditions (*Table 5.8* and *5.10, lower panels*).

Table 5.10. *Productivity parameters percentage of Arabidopsis plants untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field capacity conditions (FC) and progressive drought (PD).*

FC

	Tot_weight (mg)	**1,000_seeds**	**Tot_number**
C	100%	100%	100%
GT	113%	100%	110%
BF	105%	100%	106%
ST	103%	90%	109%

PD

	Tot_weight (mg)	**1,000_seeds**	**Tot_number**
C	100%	100%	100%
GT	62%	91%	68%
BF	74%	86%	85%
ST	46%	109%	74%

The content of fatty acids was analyzed to estimate the biostimulant effect on the Arabidopsis seeds, being a Brassicaceae, and the results are presented in *Table 5.10*. The most abundant fatty acids in Arabidopsis seeds were linoleic acid (cis-18:2), oleic acid (cis-18:2), and arachidic acid (C20:0). The foliar application with biostimulants modified the content of fatty acids in Arabidopsis seeds, but the effect depended on the biostimulant and growth conditions. For example, the seeds from the ST-treated plants decreased both saturated (SFA) and unsaturated fatty acids (UFA) under FC conditions. However, they maintained similar content of fatty acids compared to the control under PD conditions. Contrarily, BF slightly increased the content of fatty acids in the treated plants under FC, but it reduced their levels under PD, except in the case of gondoic acid (C20:1), which doubled the control values *(Table 5.11).*

Table 5.11. *Content of fatty acid methyl esters (FAMEs), [methyl erucate (C22:1), methyl heneicosanoate (C21:0), methyl arachidate (C20:0),), gondoic acid (C20:1), cis-11,14-eicosadienoic acid methyl ester (C20:2), methyl stearate (C18:0), cis-9-Oleic acid methyl ester (C18:1), methyl linoleate (C18:2), methyl palmitate (C16:0), methyl palmitoleate (C16:1)] [average ± standard deviation (SD] in Arabidopsis seeds from plants plants untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field capacity conditions (FC) and progressive drought (PD).*

		C22:1	C21:0	C20:0	C20:1	C20:2	C18:0	cis-C18:1	cis-C18:2	C16:0	C16:1
FC	C	0.2 ± 0.1	0.6 ± 0.1	6.7 ± 0.9	0.7 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	8.9 ± 1.1	18.0 ± 1.8	1.5 ± 0.2	0.1 ± 0.0
	GT	0.1 ± 0.0	0.5 ± 0.1	5.6 ± 0.4	0.5 ± 0.1	0.9 ± 0.1	1.1 ± 0.0	7.8 ± 0.5	16.9 ± 0.7	1.5 ± 0.1	0.1 ± 0.0
	BF	0.2 ± 0.0	0.7 ± 0.1	7.7 ± 0.9	0.8 ± 0.1	1.1 ± 0.1	1.4 ± 0.1	9.0 ± 1.0	19.2 ± 2.0	1.6 ± 0.2	0.1 ± 0.0
	ST	0.1 ± 0.1	0.3 ± 0.1	3.8 ± 0.9	0.4 ± 0.2	0.6 ± 0.1	0.8 ± 0.2	5.5 ± 1.1	11.9 ± 2.0	1.0 ± 0.2	0.1± 0.0
PD	C	0.1 ± 0.0	0.4 ± 0.1	5.5 ± 0.7	0.3 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	7.3 ± 1.0	14.6 ± 1.8	1.2 ± 0.2	0.1 ± 0.0
	GT	0.0 ± 0.0	0.3 ± 0.0	3.8 ± 0.3	0.2 ± 0.0	0.7 ± 0.1	0.9 ± 0.1	5.9 ± 0.5	13.4 ± 0.7	1.2 ± 0.1	0.1 ± 0.0
	BF	0.0 ± 0.0	0.3 ± 0.1	3.6 ± 0.7	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	5.7 ± 0.8	10.8 ± 1.4	0.9 ± 0.1	0.0 ± 0.0
	ST	0.1 ± 0.0	0.4 ± 0.0	4.9 ± 0.5	0.3 ± 0.3	0.7 ± 0.1	1.0 ± 0.1	6.8 ± 0.5	13.8 ± 0.8	1.2 ± 0.1	0.1 ± 0.0

5.4.4.1 MULTIVARIATE STATISTICAL ANALYSIS OF THE PRODUCTION AND QUALITY CHANGES

A multivariate statistical analysis was carried out to visualize better and simplify the result regarding yield-related parameters. The production parameters (Tot_number, Tot_weight, and weight per 1,000_seeds) and the fatty acids content obtained by GC-MS for each biostimulant, and growth conditions were evaluated together. Two PCAs were performed, one under FC and another under PD (*Fig. 5.15* and *5.16).* The PC1 and PC2 accumulated 99.65% and 96.83% of the total variance of the experimental model for FC and PD conditions, respectively.

As can be seen in *Fig. 5.15*, under FC, control and BF-treated plants presented a positive correlation with the accumulation of UFA such as gondoic acid (C20:1), erucic acid (C22:1), and concrete SFA such as heneicosanoic acid (C21:0) and arachidic acid (C20:0). They also presented the most reduced number of seeds per plant (Tot_number). However, GT was the biostimulant that most improved yield-related parameters in Arabidopsis *Fig. 5.15*.

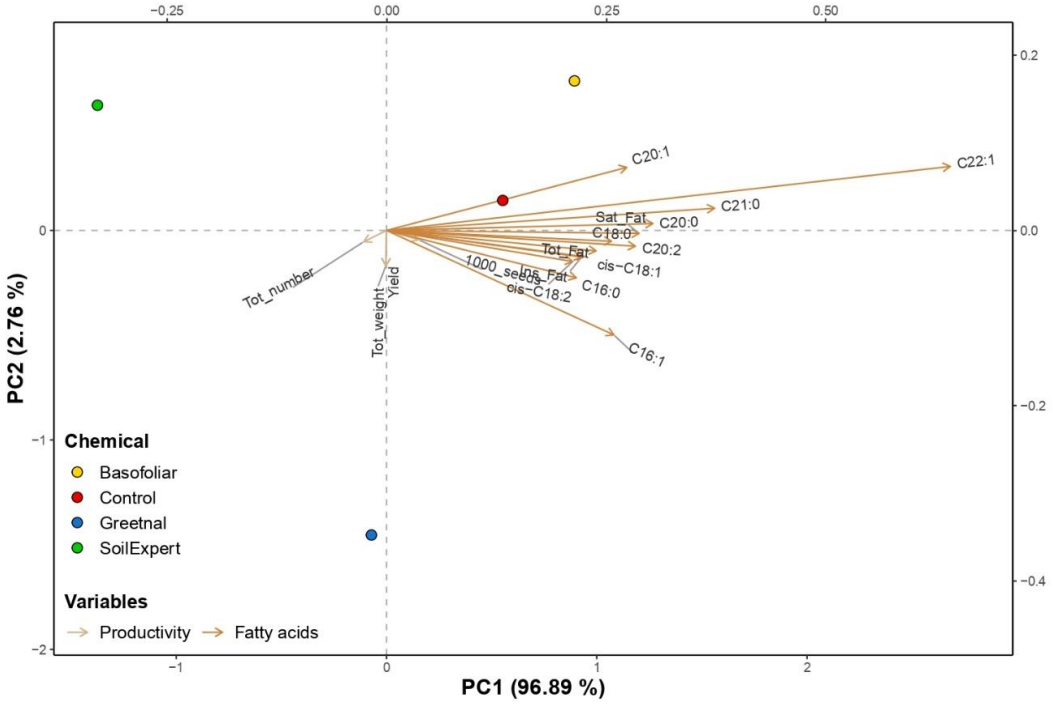


Figure 5.15. *Principal component analysis (PCA) based on fatty acid content and yield-related parameters in Arabidopsis plants untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under optimal irrigation conditions (FC).*

Under PD, the production and fatty acid content changed among treatments *(Fig. 5.16)*. For example, BF positively correlated with gondoic acid (C20:1), but it was inversely correlated to the Tot_number and plant yield, according to PC1. The biostimulant GT also showed an opposite relationship with the Tot_number and the yield according to PC2. However, ST-treated plants were closely located to the controls and presented a positive correlation with almost all fatty acids and the weight of 1,000 seeds (1,000_seeds).

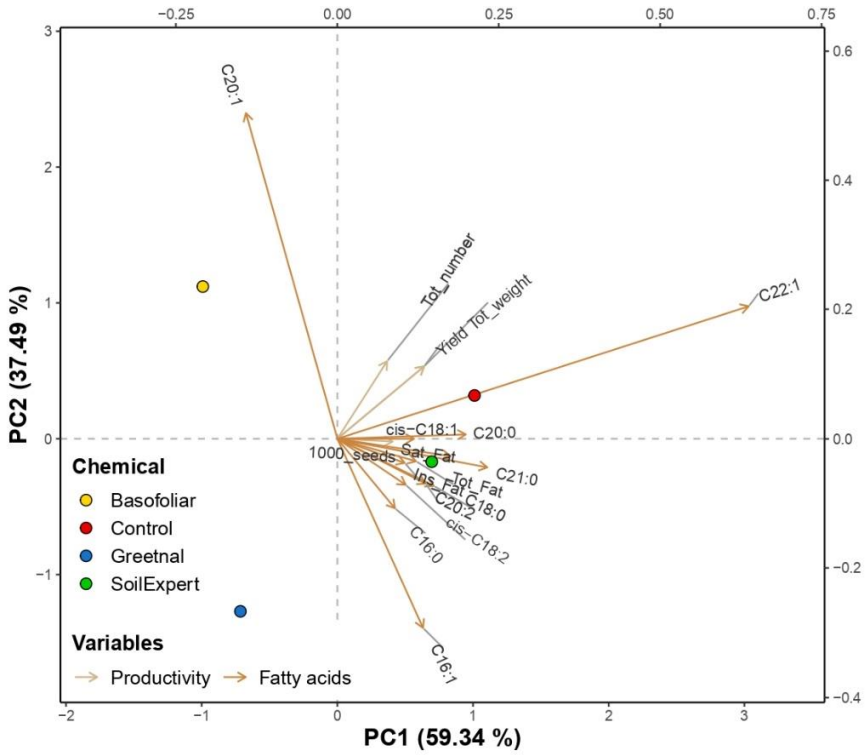


Figure 5.16. *Principal component analysis (PCA) based on fatty acid content and yield-related parameters in Arabidopsis plants untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under progressive drought (PD).*

In summary, it can be said that under FC, BF and control produced seeds with higher quality (higher fatty acid content), whereas GT treatment improved the quantity with more number of seeds per plant. However, the biostimulant application reduced the quality and quantity of Arabidopsis plants under PD.

Finally, a matrix based on Pearson´s Correlation was performed, and the results showed that no correlation existed between the quality and quantity parameters analyzed (*Fig. 5.17).* Besides, Tot_number, Tot_weight, and yield per plant showed a negative correlation with the weight of 1,000 seeds, demonstrating that the biostimulant-increased yield per plant has lighter seeds as a penalty.

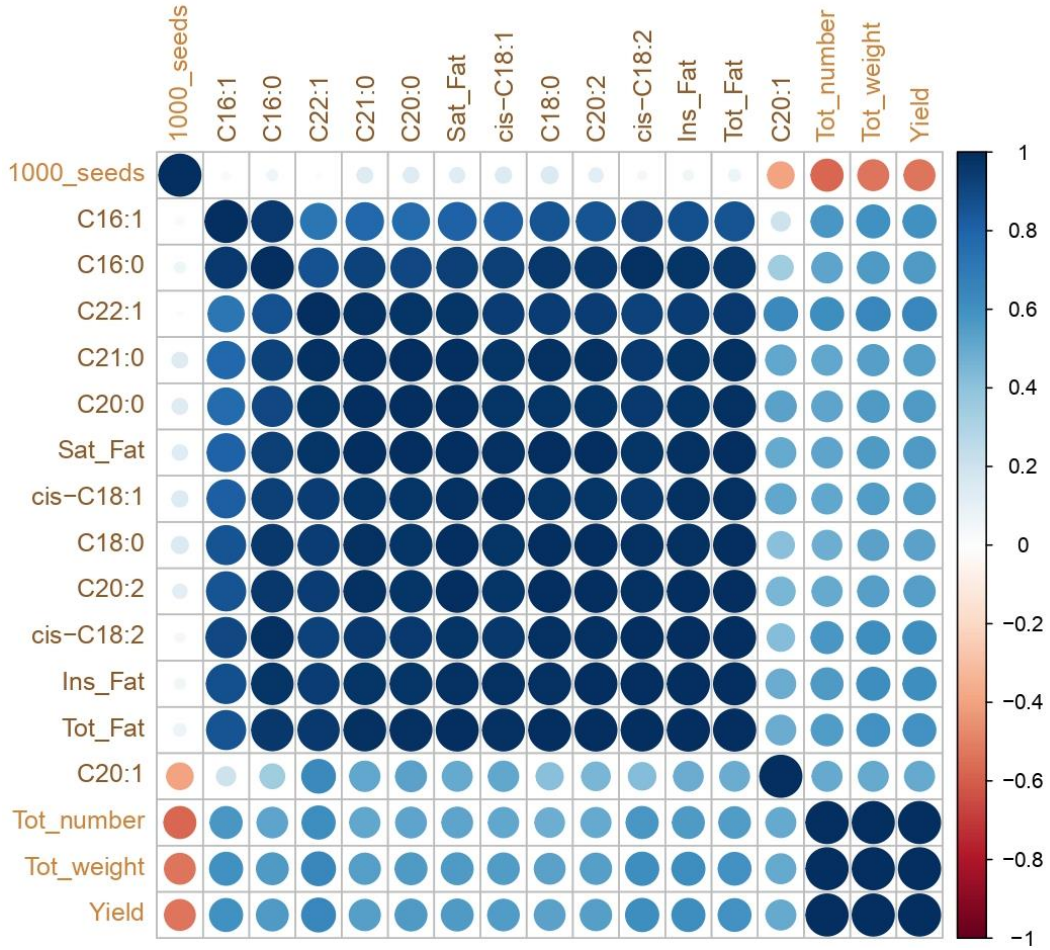


Figure 5.17. *Correlation matrix based on Pearson´s Correlation for the quantitative and qualitative parameters of Arabidopsis seeds.*

5.4.5 PLANT BIOSTIMULANTS CHARACTERISATION INDEX (PBC) FOR BIOSTIMULANTS MODE OF ACTION

Ugena et al. (2018)[404] developed an PBC index PBC to simplify the characterization of different biostimulants. This approach ended with a positive or negative value that permits the classification of the biostimulant as a growth promoter or inhibitor under optimal conditions or stress alleviator or inductor under stress conditions. The PBC index represents the sum of all values obtained for the parallel coordinates plot described in section 5.3.9.

Several morphological, physiological, and biochemical parameters as fresh weight (FW), dry weight (DW), biomass, green pixels, relative water content (RWC), foliar temperature (FTemp), maximum photosystem II (PSII) quantum yield (QY_max), steady-state non-photochemical quenching (NPQ_Lss), PSII quantum yield in light-adapted steady-state (F_v/F_m_Lss), and fluorescence decline ratio in steady-state (Rfd_Lss), instantaneous net photosynthesis (A), internal foliar CO_2 concentration in the substomatic cavities (Ci), stomal conductance (gs), instant foliar transpiration (E), instantaneous water efficiency (WUE), and total active osmolytes were used for the PBC index to characterize better the biostimulant mechanism of action under moderate and severe drought conditions. When leaf temperature and steady-state non-photochemical quenching were included, opposite values of log2 ratio were used due to their negative connotation to the plant tolerance.

Although the biostimulant treatments and growth conditions did not influence the chlorophyll fluorescence and gas exchange parameters under moderate stress conditions (after the first foliar application- 32 DAS), they were included in the calculation of the PBC index together with the FW, DW, biomass (%), green pixels, RWC_{t32}, FTemp, and total metabolites.

Under FC conditions, equivalent biomass production was presented in all biostimulant treatments, despite greater or lesser fresh or dry weight *(Fig. 5.18A*). The main differences between treatments were found in parameters such as green pixels, RWC, and mainly metabolite content. GT and BF-treated plants showed a smaller leaf area and higher DW, biomass, and metabolite content.

Under moderate drought (*Fig. 5.18B*), the biostimulant application on *Arabidopsis thaliana* seedlings did not improve the RWC and the fluorescence-related parameters. Only the biomass

represented as DW/FW ratio was improved in the treated plants, mainly with BF, compared to controls (*Fig. 5.18B*).

When *Arabidopsis thaliana* seedlings were subjected to severe drought conditions, the gas exchange parameters showed the most relevant changes. Under FC conditions (*Fig. 5.19A*), seedlings treated with ST and BF improved their biomass production compared to the control treatment. Besides, all treated plants improved the WUE more than controls, mainly due to a better A and lower E (*Fig. 5.19A.*).

Under PD one day after the second foliar application (at 39 DAS) (*Fig. 5.19B*), an evident negative effect of the foliar application with biostimulants was observed on the chlorophyll fluorescence and gas exchange parameters. However, the morphological parameter rosette area as green pixels and RWC (%) were improved in the *Ascophyllum Nodosum*-based biostimulants but not in BF-treated plants. However, only ST improved the FW and DW of the rosette. BF-treated plants reduced heat dissipation but increased DW and biomass production.

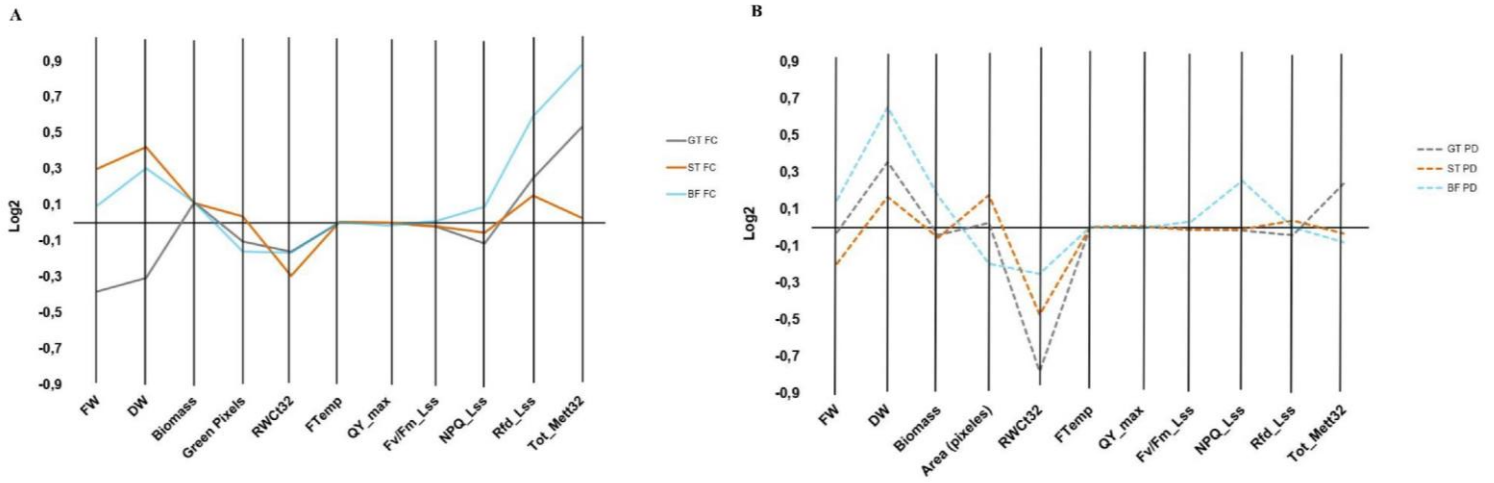
Regarding seed production, the parallel coordinate plots corroborate the previously observed data in which all biostimulants increased the Tot_weight and Tot_number of seeds per plant (*Fig. 5.20*A). However, seed production was inhibited under PD. Only ST treatment improved the weight of 1,000 seeds.

Total SFA and UFA have been used for the PBC index calculation regarding the quality of the seeds. The seeds from the BF-treatment plants were rich in SFA and UFA under FC, mainly characterized by higher UFA content (*Fig. 5.20*A). However, such treatment inhibited fatty acid synthesis when seedlings were grown under stress conditions (*Fig. 5.20*B). However, the seeds from GT and ST-treated plants presented lower fatty acids content under FC and PD.

As shown in *Fig. 5.18C*, the PBC index related to the vegetative phase of Arabidopsis showed that under FC the application of BF and ST provided positive values under moderate drought. This result indicated that they are growth promotors, with BF as the best biostimulant. In addition, BF was the only biostimulant showing positive values under moderate drought with the PD regime, acting as a stress alleviator.

Under severe stress conditions (39 DAS), only the ST-treated *Arabidopsis thaliana* seedlings showed a positive PBC index under FC conditions, keeping the growth promotor effect observed under moderate stress *(Fig. 5.19C*). However, the biostimulants behaved as stress inductors.

Finally, the PBC index on production parameters showed that only the GT and BT-treated plants promoted the yield under FC. However, the biostimulant application negatively affected the quality and quantity of Arabidopsis seeds under PD (*Fig. 5.20C).*

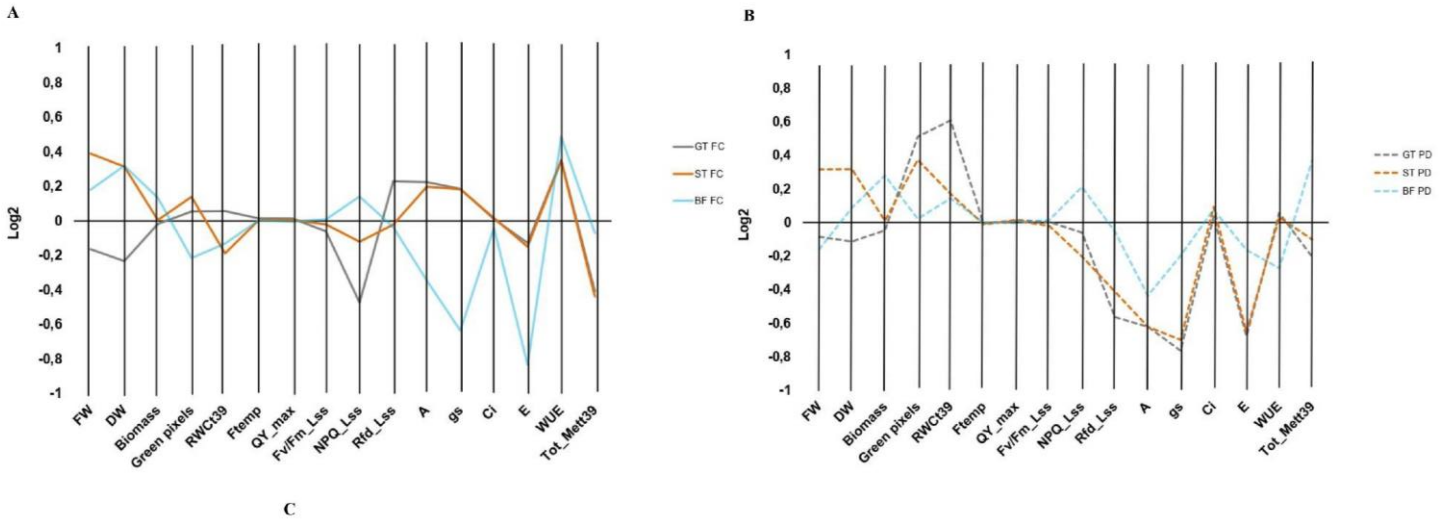


C

PBC Index for the vegetative stage at 32 DAS

	FC	PD
GT	-0.2	-0.3
BF	1.75	0.74
ST	0.69	-0.4

Figure 5.18. *Parallel coordinate plot representing the morphological, physiological, and biochemical parameters in Arabidopsis plants untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field soil capacity (FC, A) or progressive drought (PD, B) under moderate drought stress (32 DAS) and plant biostimulant Characterization (PBC) Index related to the control under moderate conditions (C) based on physiological parameters.*

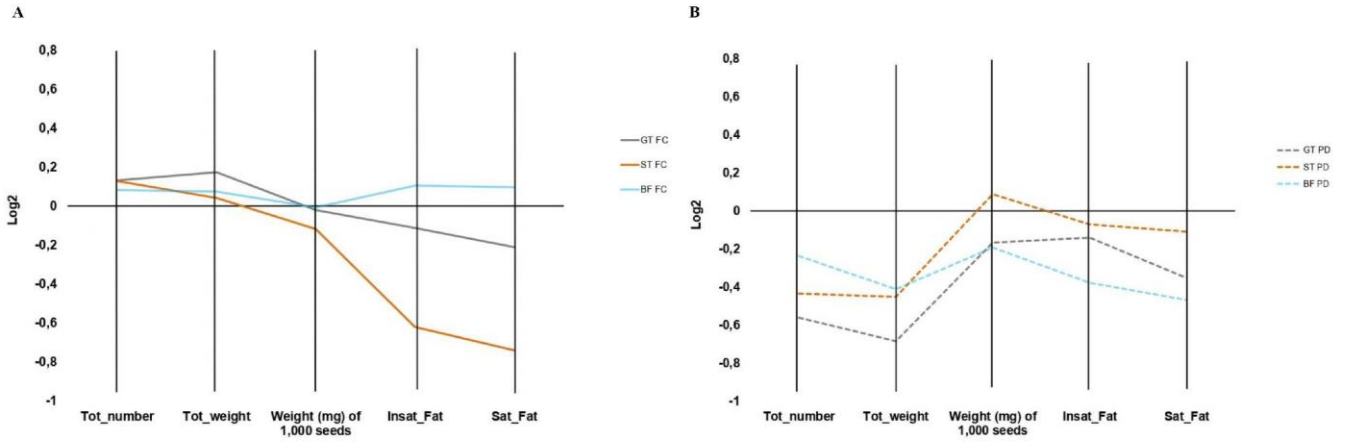


C

PBC Index for the vegetative stage at 39 DAS

	FC	PD
GT	-0.33	-1.91
BF	-1.03	-0.08
ST	0.67	-1.40

Figure 5.19. *Parallel coordinate plot representing the morphological, physiological, and biochemical parameters in Arabidopsis plants untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field soil capacity (FC, A) or progressive drought (PD, B) under severe drought stress (39 DAS) and plant biostimulant Characterization (PBC) Index related to the control under severe conditions (C) based on physiological parameters.*



C

PBC Index for production

	FC	PD
GT	0.12	-1.65
BF	0.25	-1.26
ST	-0.63	-0.89

Figure 5.20. *Parallel coordinate plot based on yield related parameters in Arabidopsis plants untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field soil capacity (FC, A) or progressive drought (PD, B) and plant biostimulant Characterization (PBC) Index related to the control under moderate conditions (C) based on yield parameters.*

5.5 DISCUSSION

Drought tolerance is a highly complicated phenomenon associated with many physiological changes in the plant, including stomatal regulation, root system, hormonal balance, antioxidant defense system, osmotic adjustment, and maintenance of tissue water content parameters[465]. Plant tolerance to drought has been classified into three main strategies depending on the physiological changes induced in the response: scape, avoidance, and tolerance[466]. These strategies usually combine different responses that allow the plant to deal with the stress[465]. For many years, the community of breeders and researchers has mainly been focused on increasing plant stress tolerance. In this context, biostimulants have been reported as an alternative to improve stress tolerance in plants[426]. Their application has been reported to improve the morphology and physiology of many species, from model plants like Arabidopsis to crops such as tomato and lettuce grown under different abiotic stress[426]. These studies were also focused on characterizing them and uncovering their mechanism of action. However, due to the complexity of these products, it is not possible to determine them easily. The use of HTP approaches allows a characterization due to their complexity. We used these approaches to characterize and compare more efficiently three commercial biostimulants, two seaweed-based products, and one substance rich in AAs. As a general result, we observed that they changed the morphology, physiology, and metabolism of Arabidopsis plants due to the type of biostimulant, the growth condition, and the stress intensity.

First, our work demonstrated that a simple foliar application was enough to decrease the rosette RWC, as observed in chapter IV. These results were evident in *Table 5.3*, in which the foliar temperature was reduced for RWC_{t32} (after the first foliar application) and RWC_{t39} (after the second foliar application) compared to RWC_{t27} (before treatment) under FC conditions. The RWC reduction happened, including in the control plants treated with water. It shows that whatever action on the plant induces physiological changes.

Going further in the biostimulant characterization and using the PBC index as a reference (*Fig. 5.18C* and *5.19C*), we showed that under optimal growth conditions, the Arabidopsis seedlings treated with BF and ST, but not GT, acted as plant growth promotors in early developmental stages (32 DAS) and after one foliar application. However, only ST remained a growth promotor after a second foliar application (39 DAS), whereas BF became a growth inhibitor and induced chlorosis in

the plant (*Fig. 5.2*). The multivariate statistical analysis always grouped the GT and ST biostimulants, so we assumed they activated a similar mechanism of action. However, going deeper into the comparison, we observed that they differed in metabolite content (**Annex IX**, *Table IX.2* to *IX.4*). For example, ST-treated plants have higher levels of GABA and BABA than GT-treated plants under FC conditions. Our analysis showed that GABA and BABA levels correlated with the FW and DW under FC conditions (*Fig. 5.9* and *5.12*). GABA is a non-protein amino acid that acts as a plant signal molecule. In general, it has been related to plant stress response and tolerance. The exogenous GABA application has also improved plant growth and photosynthesis performance in maize seedlings under non-stressful conditions[196].

Regarding BABA, this is also a non-protein amino acid mainly related to plant defense[199]. However, its application has been described as having adverse effects on plant growth, but this effect is concentration-dependent[202]. Altogether, we think that the accumulation of GABA and BABA in ST-treated Arabidopsis seedlings under optimal conditions (FC) could be the reason for better growth.

BF-treated plants presented positive values of the PBC index at 32 DAS but not at 39 DAS under FC conditions. The plants treated with this biostimulant presented higher biomass production, FW and DW, WUE, and the highest accumulation of compatible solutes *(Table 5.2).* The beneficial effect of exogenous application of amino acid-based biostimulants was reported by Koukounaras *et al.* (2013)[467] and Popko *et al.* (2018)[468]. They reported a significant increase in the yield of treated tomatoes and wheat. In our study, the foliar application of BF improved the quality and quantity of Arabidopsis seeds under optimal growth conditions. However, the second foliar application negatively affected the plant and induced chlorosis. This fact did not condition the Arabidopsis final yield. One explanation can be that the BF-treated plants showed the best WUE under FC conditions, despite reduced gas exchange and chlorosis in the leaves. In this regard, we must consider that WUE is expressed as the capacity of a crop to produce biomass per unit of water evapotranspiration and, hence, a relevant component of yield[412].

Regarding water stress, many works focused on characterizing biostimulants, especially AN seaweed-based extracts, reported that their application induced many physiological changes in the plant, including stomatal closure as a protection mechanism[395,469-472]. In our study, the foliar application of biostimulants also reduced the gas exchange parameters compared to the control under severe stress conditions. To avoid water losses, plants induce stomatal closure, which lowers

the availability of taken CO_2 and the photosynthesis rate[473]. The plants activate other metabolism pathways to compensate for the photosynthesis rate reduction. The upregulation of the Glu metabolism and concretely the GABA shunt increases the carbon flux into the tricarboxylic acid cycle to provide carbon skeletons that maintain normal cellular metabolism when carbon availability is reduced[194]. Thus, the conversion of Glu to GABA (defined as GABA/Glu ratio) has been reported as a good marker of water stress tolerance. However, in our work, the foliar application with biostimulants reduced this ratio compared to the controls, especially in BF-treated plants. Besides, the stomata closure also increased the leaf temperature[474,475]. It is worth mentioning that the biostimulant-treated plants also reduced the WUE efficiency and the plant yield. Altogether, we concluded that the foliar application with the three selected biostimulants intensifies the water stress effect and compromises the final yield in Arabidopsis. However, this effect could be due to the concentration o times of the application, so positive effects were observed after the first foliar application.

It was clear that the foliar application of biostimulant induced stress in the plants. This stress was positive (eustress) or negative (distress) depending on the biostimulant and the growth conditions. It is well-known that plants develop different response mechanisms in response to stress. These mechanisms can be based on synthesizing compatible solutes such as Pro, Asp, and Glu, among others[476-478]. Pro is the most studied compatible solutes[479-483] due to its role as a protector against the accumulation of ROS[395,477,484]. Many studies have reported Pro accumulation in plant species such as chickpea[485], fennel[486], wheat[487], sunflower[488], purple corn[489], or barley[490] under drought stress. In addition, the exogenous application of AN seaweed-based products has been related to a higher Pro synthesis in bean plants[491]. In our study, the exogenous application of ST and GT did not increase the Pro accumulation over the controls. However, the exogenous application of BF induced its accumulation in Arabidopsis under FC and PD conditions at 39 DAS (severe stress). BF-treated plants also accumulated higher Asn levels under severe stress conditions. Asn accumulation may also be an indirect result of the restriction of protein synthesis under stress conditions[492]. The accumulation of aromatic AAs (Phe, Trp, and Tyr) also occurs typically when the plants cannot reduce ROS[493,494] or due to protein degradation[493,495] induced by the stress. However, no changes in Phe and Trp were observed in our work, but Tyr was significantly accumulated in BF-treated plants. Considering that the BF-treated plants also presented chlorosis in the leaves under both growth conditions but with more intensity under severe drought, we could conclude that the accumulation

of Pro, Asn, and Tyr indicates the stress intensity under which the plant is subjected rather than the tolerance mechanism in Arabidopsis plants treated with biostimulants.

The Arabidopsis seedling sprayed with BF also accumulated higher Acet levels under FC and PD conditions (*Fig. 5.2* and *Table IX.4* of **Annex IX**). Under drought conditions, plants transform pyruvate to Acet, which plays an essential role in activating the Jasmonate signaling pathway in plants to improve drought tolerance[496]. Thus, applying amino acid-rich biostimulants could activate plant response under the two studied growth conditions. However, only under FC conditions, the Acet accumulation could help the plant grow better and be more efficient, ending with a higher final yield. Interestingly, under PD conditions, these plants improved the RWC, DW, and final biomass but reduced the gas exchange parameters and WUE, pointing to more efficient osmoregulation as a strategy to deal with the stress. This way, these plants maintained the growth and water balance but reduced their efficiency affecting the plant production.

Finally, the biostimulant effect on the seed quality was evaluated through the seeds oil. Among them, fatty acids are an essential source of human nutrition. The primary source of fatty acids in edible oils is based on palmitate (C16:0), stearate (C18:0), oleate (C18:1), linoleate (C18:2) and α-linoleate (C18:3)[497]. Only palmitate and stearate are saturated chains whose contribution to the human diet increases the risk of cardiovascular diseases[498-500]. These fatty acids and arachidic acid (C20:0) are abundant in soybean, sunflower, and even *Arabidopsis thaliana* seeds[497]. The oilseeds content of crops varied depending on the genotype and the environment[501]. Although there are few studies about the biochemistry and metabolism of oilseeds, their accumulation in plants subjected to abiotic stresses has recently been studied[502] and suggested as essential players regulating plant abiotic stress tolerance[503,504]. Concretely, they showed that the increase in unsaturated or derived poly-linoleate fatty acids (C18:3) content could modulate transduction signal pathways caused by abiotic stress since it reduces structural and/or functional membrane damages caused by the stress[504-506]. Some cell studies have shown an increase in fatty acids unsaturation or the ability to maintain unsaturation in *Arabidopsis thaliana* tissue membranes improved drought stress tolerance.

However, in our study, no effect or negative effect was observed regarding the fatty acid content of the seeds from Arabidopsis plants spayed with biostimulants. Only the BF-treated plants improved the fatty acid content, SFA and UFA under FC and the MUFA gondoic acid (C20:1) under PD. Hight levels of gondoic acid in the human diet have been associated with health problems, including

cardiovascular diseases in men and women[507]. Altogether, we could conclude that the characterization of biostimulants should cover the effect on plant growth and the quality and quantity of the final yield, so only the evaluation of the biostimulant effect on plant physiology is not enough to determine the adequacy of its use.

5.6 CONCLUSIONS

Fig. 5.21 shows a summary of the most significant changes found in Arabidopsis seedlings treated with different biostimulants (BF, GT, and ST) and two growth conditions: irrigated (FC) and non-irrigated (PD). The study also evaluated the plant response two times, corresponding with moderate and severe drought. Our studies indicate that applying foliar biostimulants based on AN seaweed extracts and AAs can be considered a sustainable tool to improve plant growth and agricultural productivity under optimal conditions and moderate water stress. However, they are stress inductors when Arabidopsis suffers severe stress. Only the biomass was maintained in those plants treated with BF and ST.

The biostimulant application mainly modified the growth-related and gas exchange parameters, increasing the FW and DW of the rosettes and reducing the transpiration and photosynthesis in Arabidopsis. However, they improved the water use efficiency and, in combination with their metabolic profile, could be classified hierarchically based on the level of drought to which the crop was subjected (*Fig. 5.21*). The biostimulant BF promoted plant growth and yield due to a higher accumulation of AAs such as Pro, Gly, Tyr, and Asn, among other AAs, the organic acid Acet, and the highest WUE under optimal conditions, including when its application induced chlorosis in the leaves. The two AN-based biostimulants showed similar physiological responses in the plants. However, ST but not GT improved plant growth under FC conditions, mainly due to higher GABA and BABA levels. Our results demonstrate that more profound studies that include vegetative and productive phases of the plants are needed for an adequate characterisation of the plant biostimulants. However, a minimal number of studies are focused on the application effect of these substances on plant yield.

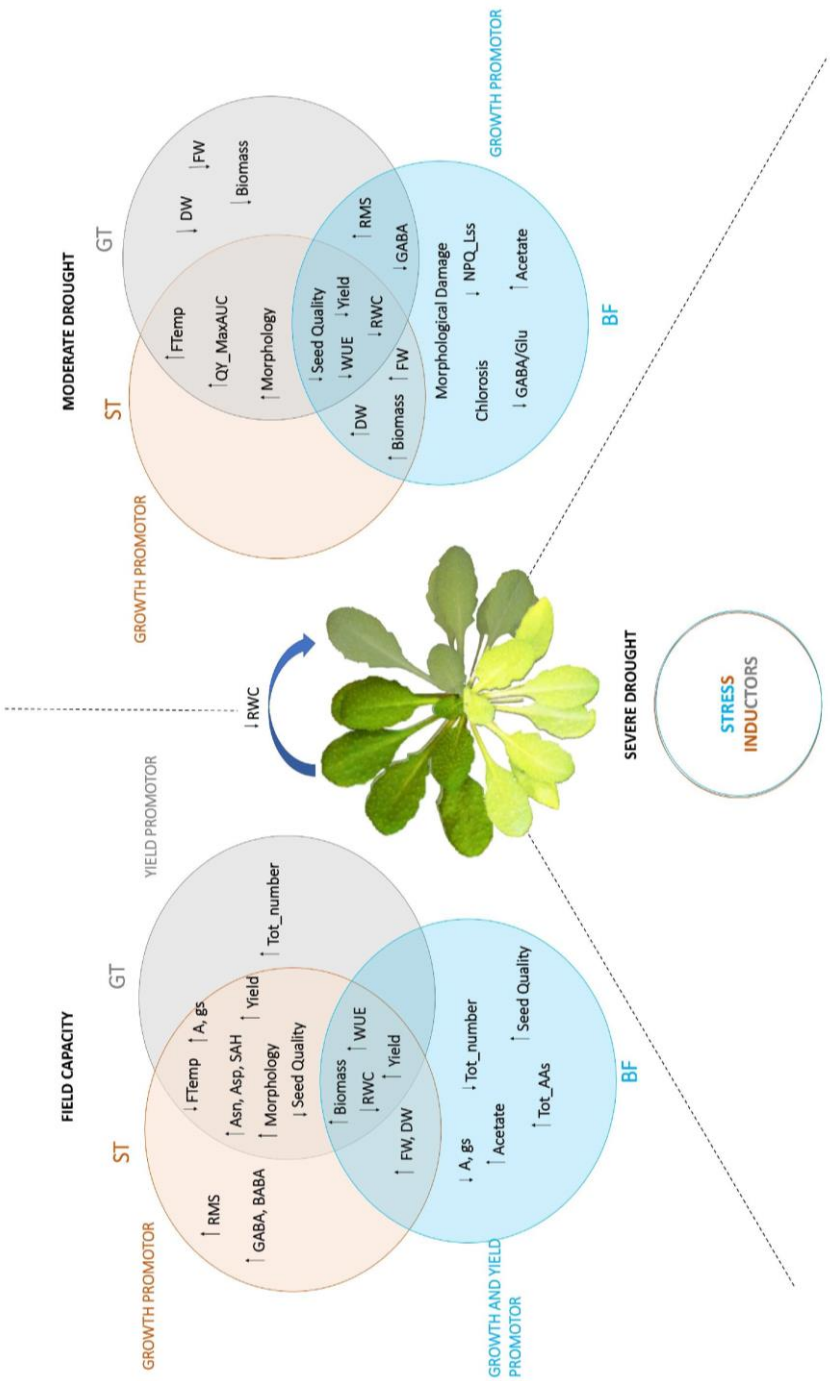


Figure 5.21. *Proposed mechanism of action in Arabidopsis plants treated with the three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] in Arabidopsis plants under field soil capacity, moderate drought or severe drought.*

Capítulo VI: Traslación biológica entre *Vitis vinífera* L. y *Arabidopsis thaliana*

6.1 TRASLACIÓN BIOLÓGICA ENTRE *VITIS VINIFERA L.* Y *ARABIDOPSIS THALIANA*

Nuestro estudio puede ser utilizado como un ejemplo de traslación biológica entre dos cultivos diferentes y en condiciones experimentales diferentes; una planta leñosa como *Vitis vinifera* estudiada en campo abierto y una planta de la familia de las brasicacea como *Arabidopsis thaliana* estudiada con herramientas innovadoras de fenotipado en condiciones controladas bajo condiciones de déficit hídrico con el fin de determinar su tolerancia, capacidad de recuperación y/o aclimatación frente al tratamiento con tres bioestimulantes comerciales de distinta naturaleza. En ambas especies se evaluaron cambios fisiológicos que se produjeron a lo largo de ciclos de estrés a corto y largo plazo, incluyendo las señales bioquímicas y sus interconexiones. De igual manera, se profundizó en la caracterización del mecanismo de acción en ambas especies incluyendo el tratamiento con un AA-no proteico (BABA) en el viñedo. Para el análisis, se utilizaron diversas herramientas estadísticas, como multi-ANOVAs y PLS-DA en el caso de *Vitis vinifera* y multi-ANOVA, PCA y matriz de correlación de Pearson en el caso de *Arabidopsis thaliana*.

En ambas especies, se observó una rápida respuesta en la fisiología y en el metabolismo primario en función del tipo de bioestimulante, condición de crecimiento e intensidad del estrés (*Fig. 6.1*). En ambos casos, la simple aplicación de los tratamientos bioestimulantes produce una disminución en el contenido de agua; RWC en el caso de Arabidopsis y LWC en el caso de *Vitis vinifera*. Sin embargo, en *Vitis vinifera*, se produce una mejora del LWC cuando el estrés fue severo (2019), mientras que en *Arabidopsis thaliana* se produjo una reducción del RWC que indica una intensificación del estrés en condiciones extremas. Así mismo, mientras que en *Vitis vinifera*, se promueve WUE bajo estrés moderado (2018) y se promueve el rendimiento y la eficacia del cultivo, en *Arabidopsis thaliana*, se mejora la WUE en condiciones optimas, pero se produce un significativo descenso del WUE, comprometiendo, de esta manera, el rendimiento vegetal. Estos resultados demostraron que la evaluación de la WUE en planta es un buen biomarcador que permitiría predecir el rendimiento final de las plantas[412].

Por otro lado, en el caso de *Vitis vinifera*, se observó que la climatología y la ubicación de las vides dentro del cultivo son determinantes en la eficacia de los tratamientos bioestimulantes. En *vitis vinifera*, todos los tratamientos bioestimulantes son capaces de mejorar la fisiología, pero únicamente bajo condiciones de sequía moderada (2018) en la subparcela A, siendo el tratamiento

con BABA el que más eficazmente actúa, puesto que además de su efecto bioestimulante actúa como inhibidor de plagas como mildiu[192]. Sin embargo, el tratamiento BF también es capaz de promover la fisiología incluso en ambientes de estrés severo (2019), pero dependiente de la localización de las vides. Sin embargo, en *Arabidopsis thaliana* la eficacia de los tratamientos depende la concentración, el numero de aplicaciones y la intendidad del estrés.

Respecto a la calidad de los productos finales, en el caso de *Vitis vinifera*, se observó una mejora de su calidad, ya que los tratamientos bioestimulantes potenciaron una disminución de los azúcares y una mejora en los ácidos orgánicos, que derivará en una mayor calidad del vino producido gracias al mantenimiento del ácido málico (*Fig. 6.1*). De todos ellos, ST es el tratamiento que más potenció la calidad de las bayas, pero no GT, incluso cuando ambas substancias se basan en el mismo material de partida (*Ascophyllum nodosum*). Sin embargo, en el caso de *Arabidopsis thaliana*, BF fue el bioestimulante que mejor funcionó como aleviador del estrés, principalmente debido a la acumulación de metabolitos osmorreguladores bajo estrés moderado. Este hecho, podría ser debido a la alta concentración de aminoacidos libres, y de ácido málico observado en el mismo. Sin embargo, este tratamiento compromete el rendimiento y la producción en condiciones extremas en ambos cultivos.

Además, en ambos cultivos se observan mecanismos de acción diferentes para los tratamientos a base de algas marinas AN. Mientras que en *Vitis vinifera*, ST promueve la acumulación de ácidos orgánicos, en *Arabidopsis thaliana* promueve la acumulación de GABA y BABA y mejora el crecimient (*Fig. 6.1*). Tampoco se observó un mecanismo semejante para las dos especies tratadas con GT. Por este motivo es de esencial atención una previa caracterización de los productos bioestimulantes en función del cultivo en el que se pretende utilizar, ya que el modo de preparación de la materia prima puede cambiar la composición bioquímica del producto final, y de esta manera, tanto la respuesta fisiológica como metabólica de la planta.

Finalmente, el uso del índice PBC permitió la simplicación y profundización en el estudio del mecanismo de acción de los bioestimulantes en ambos cultivos. En *Vitis vinifera*, todos los tratamientos son promotores del crecimiento en la subparcela A bajo condiciones moderadas, y especialmente el tratamiento con BABA potencia la produccion y protege a la planta contra enfermedades. En *Arabidopsis thaliana*, únicamente ST y BF promueven el crecimiento en estrés moderado además de que ST mantiene el crecimiento y BF inhibe el crecimiento y produce clorosis

en condiciones extremas. En conclusión, aunque hay ciertos comportamientos similares entre especies, la translación biológica entre Arabidopsis y *Vitis vinífera* fue mas visible en condiciones optimas y de estres moderado que en estrés severo, muy probablemente debido a la diferencia en tolerancia frente al estrés. Sin embargo, lo que si quedo demostrado es que la WUE en planta es un buen biomarcador que permitiría predecir el rendimiento final de las plantas[412].

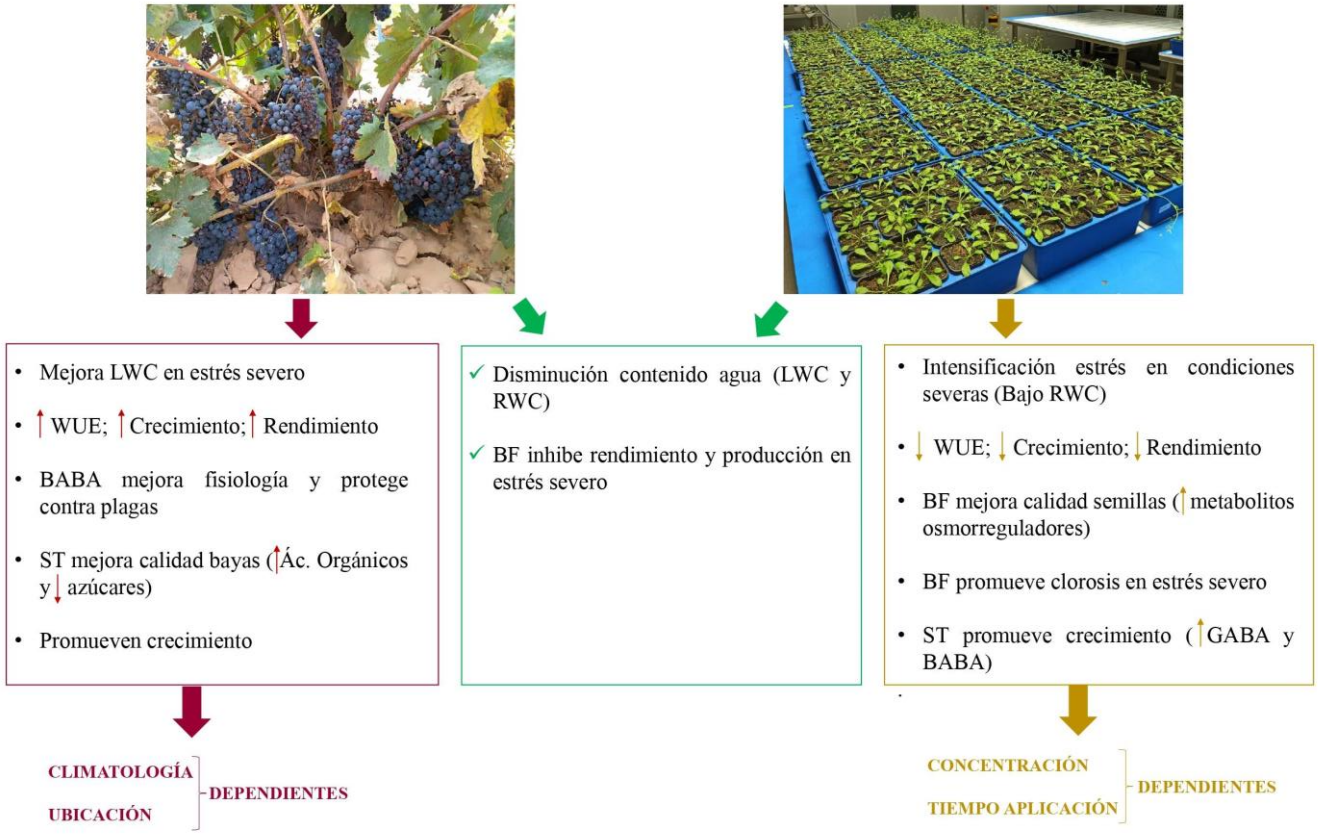


Figura 6.1. *Resúmen del mecanismo de acción propuesto para tres bioestimulantes comerciales [Greetnal (GT), Basofoliar (BF) or SoilExpert (ST)] y un aminoácido no proteico (BABA) frente a la tolerancia al estrés hídrico en* Vitis vinifera *y* Arabidopsis thaliana.

Capítulo VII: Conclusiones Generales

7.1 CONCLUSIONES GENERALES

Teniendo en cuenta el objetivo principal del presente estudio sobre el impacto que el uso de herramientas prometedoras como los bioestimulantes a base de algas marinas *Ascophyllum nodosum*, AAs vegetales y AAs no proteicos, para minimizar el impacto que los recientes cambios ambientales y el estrés hídrico tienen sobre un cultivo de *Vitis vinífera* L. se puede concluir que:

1. Se han obtenido datos sobre la presencia de carbohidratos, ácidos orgánicos y aminoácidos libres en diferentes tejidos de Vitis vinífera y Arabidopsis thaliana, que permiten extraer conclusiones sobre el estado vegetativo de la planta. Para ello se optimizaron y validaron tres metodologías analíticas basadas en cromatogragía líquida (HPLC) y acoplada a detectores de índice de refracción (RID), de matriz de diodos (DAD) y de fluorescencia (FLD).

2. Una simple aplicación foliar de agua en los cultivos induce cambios fisiológicos en las plantas, reduciendo el balance hídrico de las mismas.

3. Los tratamientos bioestimulantes producen una rápida respuesta fisiológica y bioquímica en los cultivos, pero esta depende en función del tipo de bioestimulante, condición de crecimiento e intensidad del estrés.

4. La naturaleza de los bioestimulantes no asegura una misma respuesta en la planta, ya que la manipulación y preparación industrial condiciona el perfil bioquímico del producto final

5. Las respuestas fisiológicas y metabólicas de *Vitis vinífera* variaron en función de la climatología y ubicación de las subparcelas dentro del cultivo, mostrando diferentes niveles de tolerancia y acondicionamiento frente al estrés hídrico.

6. La respuesta fisiológica en *Arabidopsis thaliana* está condicionada por la dosis, numero de tratamientos, y condiciones de crecimiento.

7. El parámetro fisiológico WUE mostró evidencias suficientes para ser utilizado como biomarcador para predecir el rendimiento y la eficacia de los cultivos, puesto que mejora la producción de biomasa y la producción final.

8. El tratamiento de *Vitis vinifera* con aminoácidos no proteicos (BABA) potencia el crecimiento y promueve la resistencia frente a plagas, aunque esta respuesta esta muy influenciada por las condiciones ambientales.

9. El perfil metabólico puede ser empleado para determinar la calidad de los productos finales en ambos cultivos, o como biomarcadores de la tolerancia, activación e intensidad del estrés hídrico.

10. El protocolo basado en métodos HTS puede facilitar la identificación del mecanismo de acción de bioestimulantes conocidos y ayudar a la identificación y su selección en función del tipo de cultivo.

11. La aplicación de bioestimulantes puede mejorar el crecimiento de la planta, la calidad y cantidad de los cultivos, aunque la respuesta depende de la combinación especie-bioestimulante.

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Annex

ANNEX I: ADDITIONAL TREATMENTS DURING THE PHENOLOGICAL STAGES AND OBSERVATIONS.

Table I.1. *Additional vineyard treatments and damages during 2018.*

Date	*Plague*	*Commercial Product*	*Dose*	*Observations*
1-3-18	Grass	PATTEN GREEN	1 L/ha	
8-5-18	Oidium	AIRUS	170 cc/ha	
9-5-18	Dust Mites	ENVIDOR	100 cc/ha	
10-5-18	Mildew	ESTUDER	1.5 kg/ha	
20-6-18	Mildew	POPPIER PRO	2 kg/ha	
21-6-18	Oidium	LUNA EXPERIENCE	400 cc/ha	Poor setting in GT, ST, and BF in Plot A and BF in Plot B. (*Fig AI.1.).*
2-7-18	Mildew	POPPIER PRO	2 kg/ha	
3-7-18	Mildew	ASBELTO	2 kg/ha	
3-7-18	Oidium	COLLIS	400 cc/ha	
17-7-18	Mildew	ELECTIS	1 L/ha	Two strong storms of 26L/h and 20L/h that affected GT and BF in Plot B *(Fig AI.2.)* and C, GT, BF, and ST in Plot A and (*Fig AI.3.).*
17-7-18	Oidium	MILORD	500 cc/ha	
8-8-18	Mildew	CALDO BORDELÉS RSR DISPERS	3 kg/ha	
25-8-18	Mildew	CALDO BORDELÉS RSR DISPERS	3 kg/ha	Mildew in Greetnal and BF in Plot B (*Fig AI.4.).*
27-8-18	Mildew	CUPROXAT 34.5	0.8 %	-Botrytis cinérea in BF (Plot A). (*Fig AI.5.).* -Powdery mildew in ST in Plot B. (*Fig AI.6.).*

Figure I.1. *Poor setting in clusters from GT, ST, and BF-treated plants in Plot A, and Basofoliar (subplot B) in 2018.*

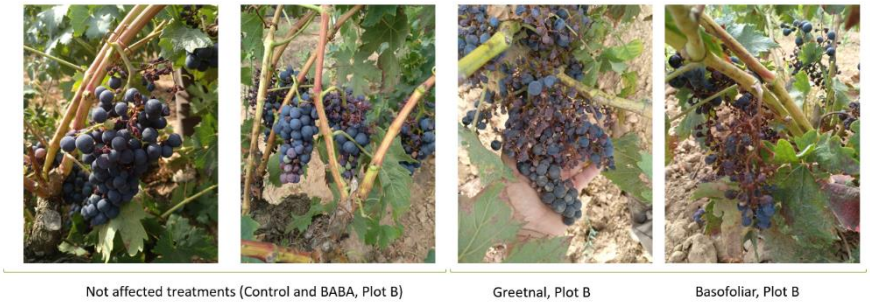

Figure I.2. *Strong storms damages in GT and BF-treated clusters (Subplot B) in 2019.*

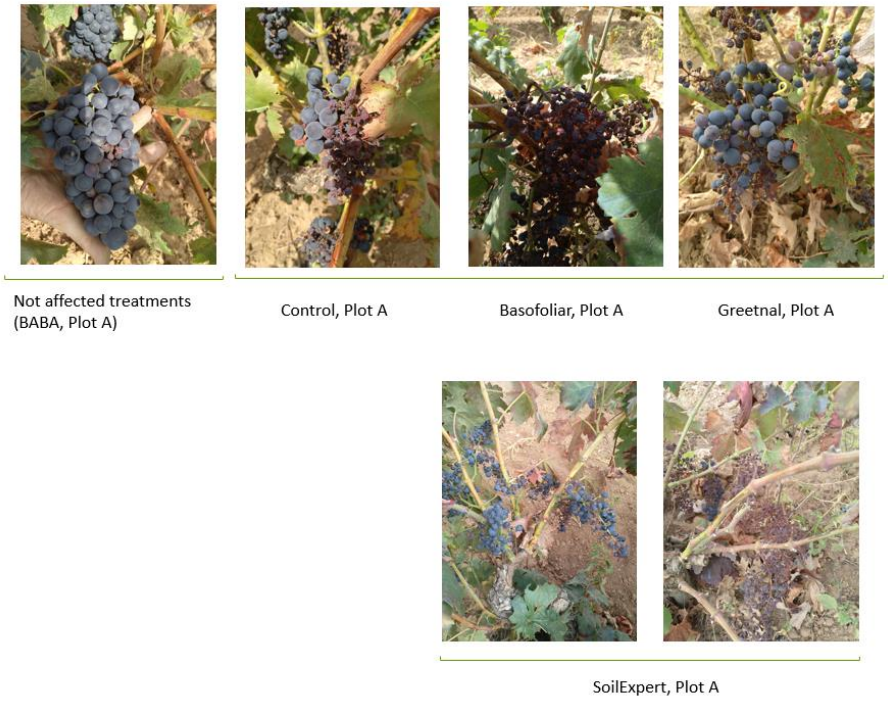

Figure I.3. *Strong storms damages in clusters from C, GT, ST and BF-treated plants (subplot A) in 2018.*

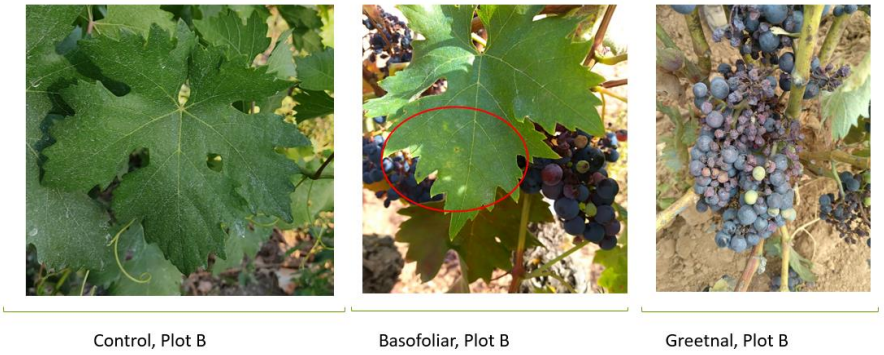

Figure I.4. *Mildew in leaves and clusters from GT and BF-treated plants (Subplot B) in 2018.*

BABA, Plot A Basofoliar, Plot A

Figure I.5. *Botrytis in clusters from BF-treated plants (Subplot A) in 2018.*

Control, Plot B SoilExpert, Plot B

Figure I.6. *Powdery mildew in clusters from ST-treated plants (Subplot B) in 2018.*

Table I.2. *Additional vineyard treatments and damages during 2019.*

Date	***Plague***	***Commercial Product***	***Dose***	***Observations***
5-3-19	Grass	ROUNDUP PLUS		
3-5-19	Dust Mites	EVIPOR	100 cc/ha	-Yesca desease in C and GT in Plot A (see *Fig AI.7.).* -Condition of *Empoasca spp.* in GT of Plot A (*Fig AI.8.).*
6-5-19	Oidium	MIOROTHIOL SPECIAL DISPERS	2 kg/ha	
11-5-19	Erinosis	EVIPOR	100 cc/ha	-Condition in ST in Plot A (*Fig AI.9.).*
13-5-19	Iron corector	HUMITEC EXTRA DRY	3 kg/ha	
29-5-19	Mildew	CAPRIF	2 kg/ha	
29-5-19	Oidium	ATTENZO STAR	200 cc/ha	
17-6-19	Mildew	MELODI CONBI	1.5 kg/ha	
17-6-19	Oidium	LUNA EXPERIENCE	400 cc/ha	
12-7-19	Mildew	MILRAD-PRO	400 cc/ha	
12-7-19	Oidium	PROSPER EC	500 cc/ha	
13-8-19	Mildew	CUPRITAL SUPER	2 kg/ha	-Hail damage in ST in Plot A (*Fig AI.10.).*

Figure I.7. *Yesca disease in leaves from C and ST-treated plants (Subplot A) in 2019.*

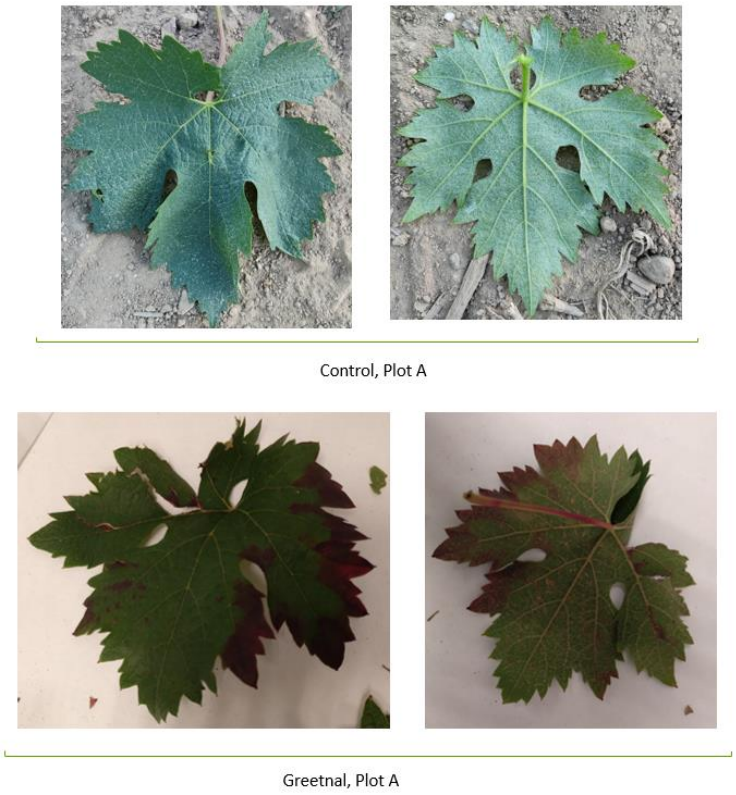

Figure I.8. *Empoasca spp. disease in leaves from GT-treated plants (Subplot A) in 2019.*

Figure I.9. *Erinosis disease in leaves from ST-treated plants (Subplot A) in 2019.*

Figure I.10. *Hail damage in clusters from ST-treated plants (Subplot A) in 2019.*

Table II.1. *Climate data recorded in the vineyard during the seasons 2018 and 2019.*

	2018				2019			
	Tmin.	**Tmax.**	**P. (mm)**	**Wind Direction**	**Tmin.**	**Tmax.**	**P. (mm)**	**Wind Direction**
Jan	-1.8	17.3	75.62	W	-2	15	47.1	SE
Feb	3.5	15.6	34.9	SE	-1.5	15.6	36.4	WNW
Mar	-2	21.6	42.5	SE	0	23.2	5.8	WNW
Apr	3	24.6	73.3	WNW	1	23.6	78.1	SE
May	5	26.7	51.2	SE	2	29.5	33.8	WNW
Jun	9	33.1	32.8	SE	7.5	40.5	42.6	WNW
Jul	14.3	32.8	70.4	W	11	37.5	29	WNW
Aug	11.7	36	-	-	11.2	34.4	14.5	S
Sep	10	32.4	31.5	SE	8.5	33	66.7	NE
Oct	1.5	25.3	39	WNW	4.8	28.4	28.6	WNW
Nov	0.8	17.6	67.8	SE	1.5	21.4	120	WNW
Dec	0.5	15.5	32.6	SE	-2.6	16	28.1	SE

N:North. S:South. W:West. E:East.

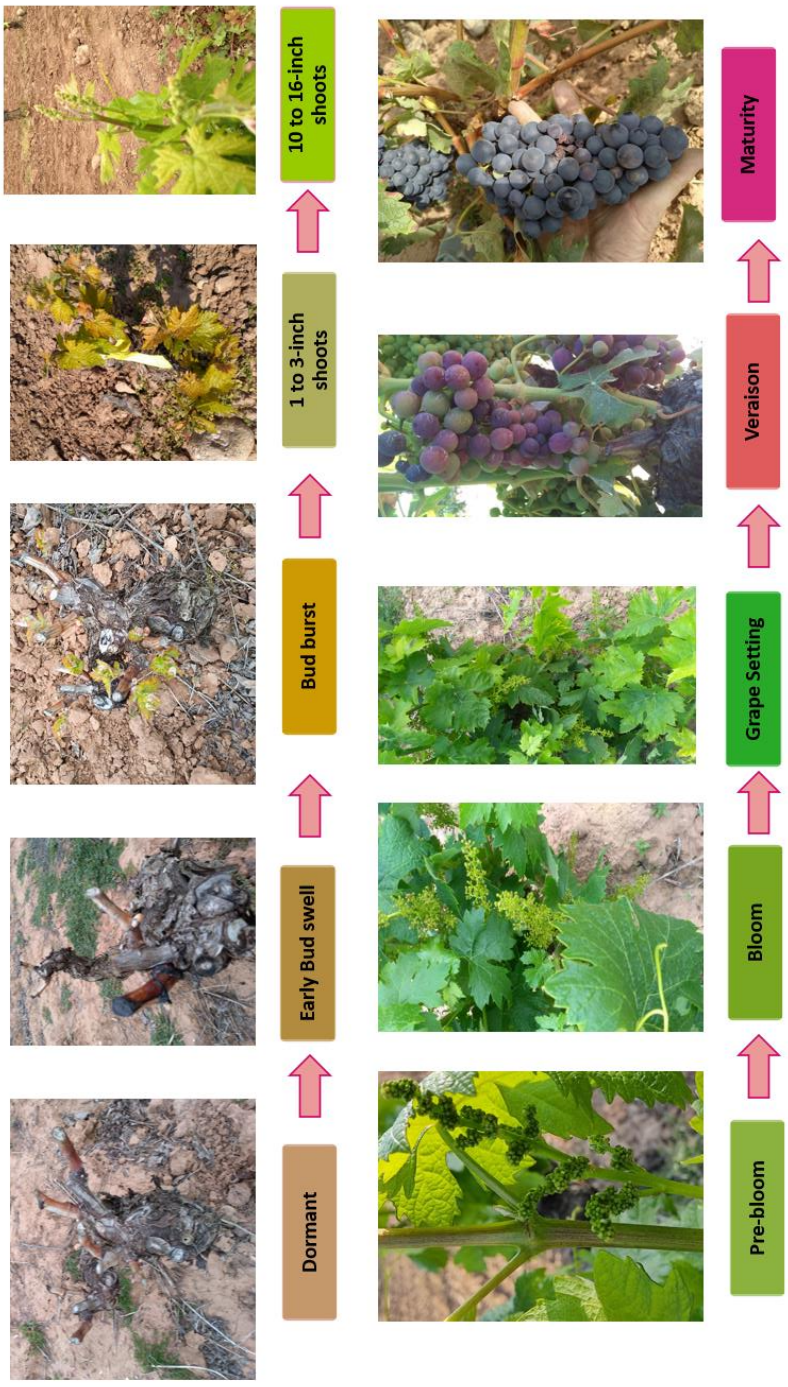

Figure II.1. *Phenological stages of the studied* Vitis vinifera *vineyard.*

Table II.2. *Dates of the phenological stages during the studied seasons.*

	2018	***2019***
BB	March, 1	March, 11
BL	June, 19	June, 8
V	August, 21	August, 6
M	September, 27	September, 21

ANNEX III: STUDY OF THE MATRIX EFFECT IN THE QUANTIFICATION OF CARBOHYDRATES USING HPLC-RID.

Table III.1. *Standard addition and external calibration curves and matrix coefficient in a pooled leaf sample of* Vitis vinifera *L.*

(mg L^{-1})	Slope S.A. ± error	Slope E.C. ± error	Matrix Effect (%)
Fructose	62.1 ± 1.5	63.1 ± 0.9	1.7
Glucose	35.0 ± 0.5	35.6 ± 0.5	1.5
Sucrose	59.2 ± 2.6	61.5 ± 0.4	3.7
Maltose	17.0 ± 0.5	17.4 ± 0.3	2.0

Table III.2. *Standard addition and external calibration curves and matrix coefficient in a pooled must of* Vitis vinifera *L.*

(mg L^{-1})	Slope S.A. ± error	Slope E.C. ± error	Matrix Effect (%)
Fructose	64.6 ± 1.4	64.6 ± 1.0	-0.0
Glucose	41.7 ± 0.7	41.1 ± 0.6	-1.5

Table III.3. *Standard addition and external calibration curves and matrix coefficient in a pooled grape of* Vitis vinifera *L.*

(mg L^{-1})	Slope S.A. ± error	Slope E.C. ± error	Matrix Effect (%)
Fructose	63.8 ± 0.6	64.6 ± 1.0	1.3
Glucose	41.6 ± 0.6	41.1 ± 0.6	-1.3

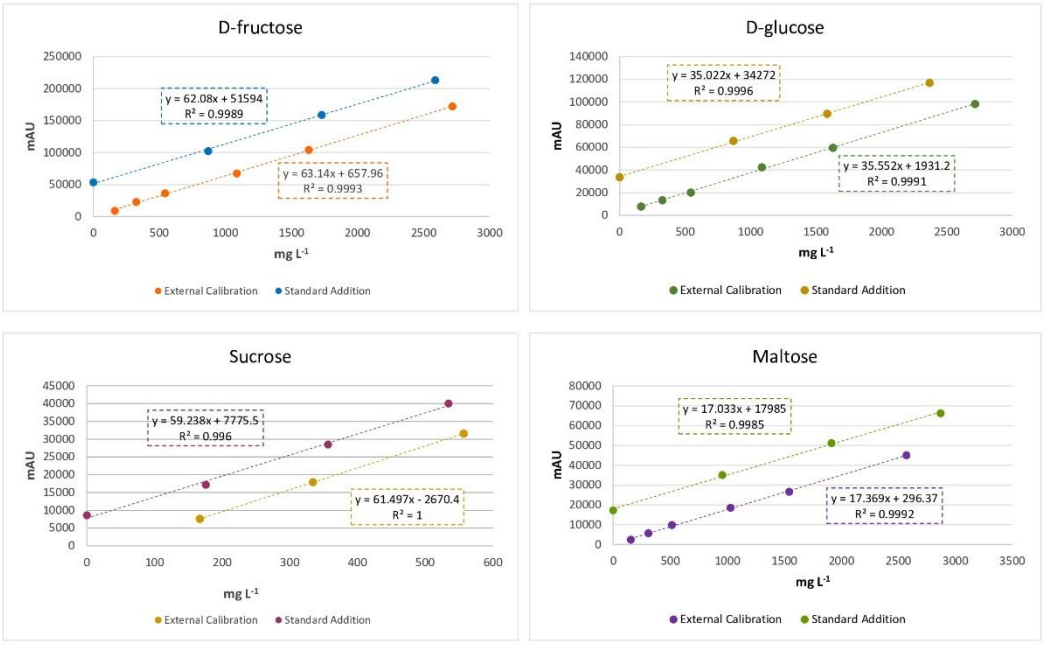

Figure III.1. *Matrix effect for Fructose (a). Glucose (b). Sucrose (c) and Maltose (d) in* Vitis vinifera L. *leaves.*

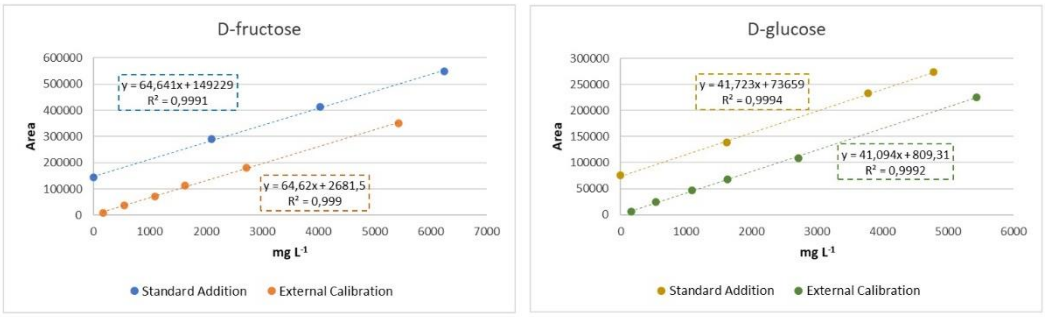

Figure III.2. *Comparison of the matrix effect for Fructose (a) and Glucose (b) in* Vitis vinifera L. *musts.*

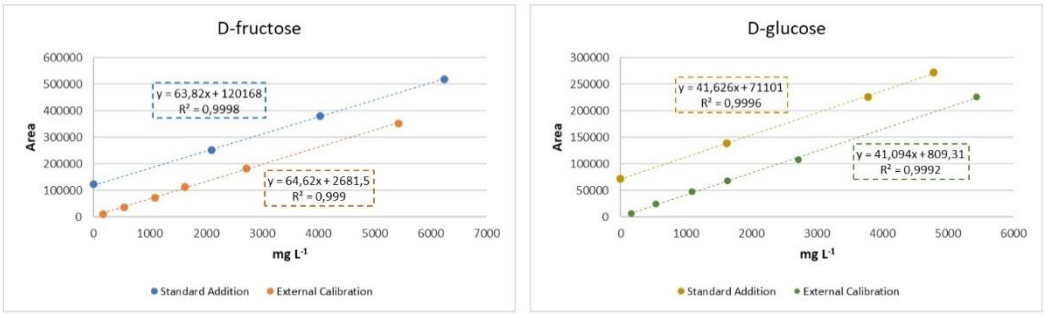

Figure III.3. *Comparison of the matrix effect for Fructose (a) and Glucose (b) in* Vitis vinifera *L. grapes.*

ANNEX IV: STUDY OF THE MATRIX EFFECT IN THE QUANTIFICATION OF ORGANIC ACIDS USING HPLC-DAD.

Table IV.1. *Standard addition and external calibration curves and matrix coefficient in a pooled leaf of* Vitis vinifera L. *for organic acid quantification.*

mg L^{-1}	Slope S.A. ± error	Slope E.C. ± error	Matrix Effect (%)
Oxalic Acid	32.4 ± 0.8	33.9 ± 0.4	4.5
Tartaric Acid	13.7 ± 0.3	13.8 ± 0.1	1.1
Malic Acid	6.8 ± 0.2	6.8 ± 0.1	-2.4
Acetic Acid	5.6 ± 0.1	5.4 ± 0.0	-2.4
Citric Acid	13.9 ± 0.2	14.2 ± 0.1	1.8
Succinic Acid	3.5 ± 0.1	3.5 ± 0.0	0.6
Fumaric Acid	2,476.2 ± 63.9	2,523.0 ± 37.0	1.9

S.A. Standard Addition method. E.C. External Calibration method.

Table IV.2. *Standard addition and external calibration curves and matrix coefficient in a pooled must of* Vitis vinifera L. *for organic acid quantification.*

mg L^{-1}	Slope S.A. ± error	Slope E.C. ± error	Matrix Effect (%)
Oxalic Acid	22.9 ± 0.6	22.4 ± 0.2	-2.0
Tartaric Acid	14.3 ± 0.6	14.2 ± 0.1	-1.1
Malic Acid	5.4 ± 0.1	5.5 ± 0.1	0.9
Acetic Acid	4.2 ± 0.1	4.1 ± 0.1	-1.5
Citric Acid	8.2 ± 0.1	8.2 ± 0.1	-0.9
Succinic Acid	12.6 ± 0.2	12.4 ± 0.1	-2.0
Fumaric Acid	2,363.4 ± 19.4	2,321.1 ± 30.0	-1.8

S.A. Standard Addition method. E.C. External Calibration method.

Table IV.3. *Standard addition and external calibration curves and matrix coefficient in a pooled grape of* Vitis vinifera L. *for organic acid quantification.*

mg L^{-1}	Slope S.A. ± error	Slope E.C. ± error	Matrix Effect (%)
Oxalic Acid	22.9 ± 0.2	22.4 ± 0.2	-2.2
Tartaric Acid	14.9 ± 0.4	14.2 ± 0.1	-4.7
Malic Acid	5.7 ± 0.1	5.6 ± 0.0	-2.3
Acetic Acid	4.3 ± 0.1	4.3 ± 0.1	-0.1
Citric Acid	7.6 ± 0.1	7.4 ± 0.1	-2.0
Succinic Acid	11.9 ± 0.5	11.3 ± 0.2	-4.8
Fumaric Acid	2,299.8 ± 87.7	2,277.6 ± 25.7	-1.0

S.A. Standard Addition method. E.C. External Calibration method.

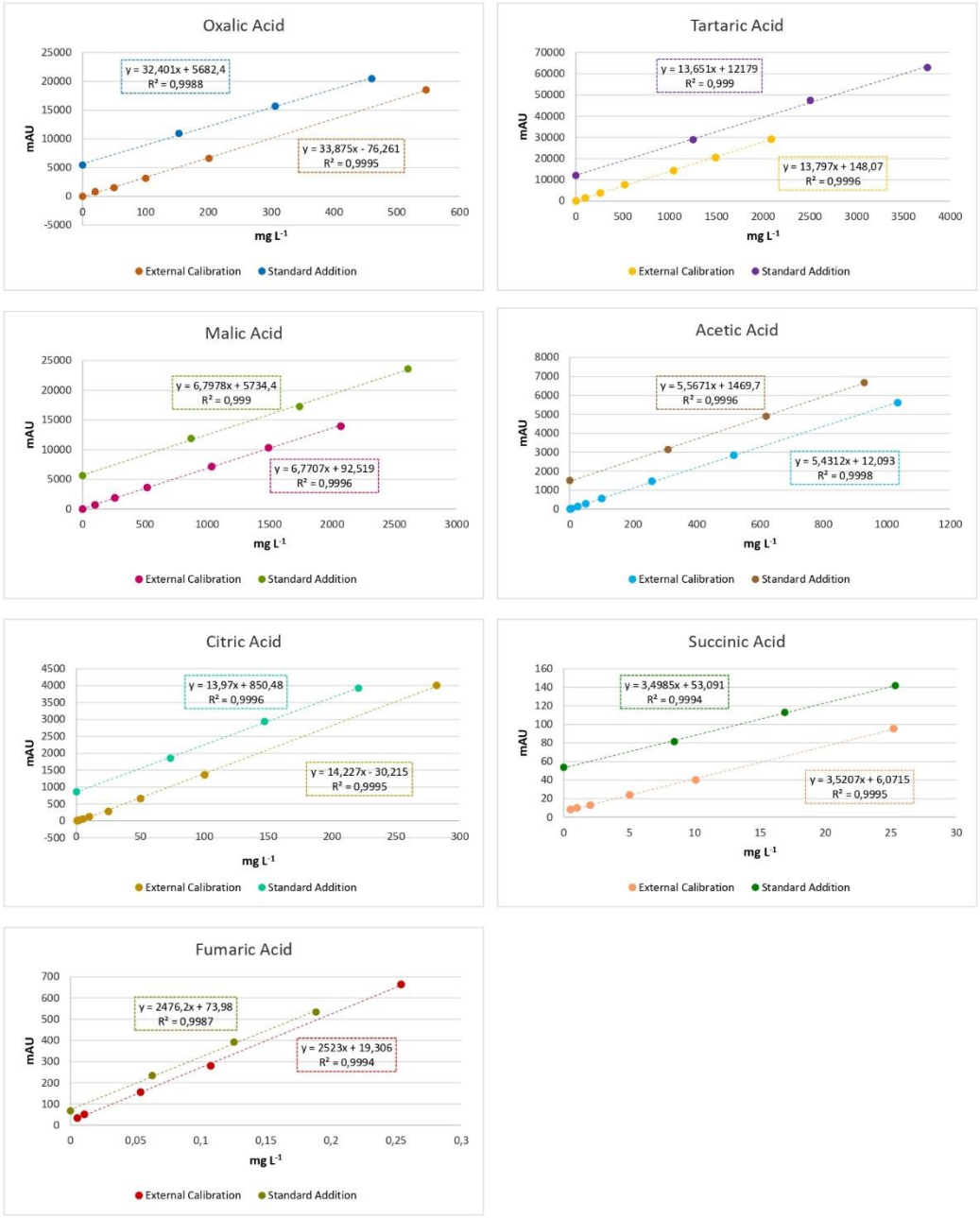

Figure IV.1. *Matrix effect for Oxalic acid (a), Tartaric acid (b), Malic acid (c), Acetic acid (d), Citric acid (e), Succinic acid (f) and Fumaric acid (g) in a pooled leaf of* Vitis vinifera *L.*

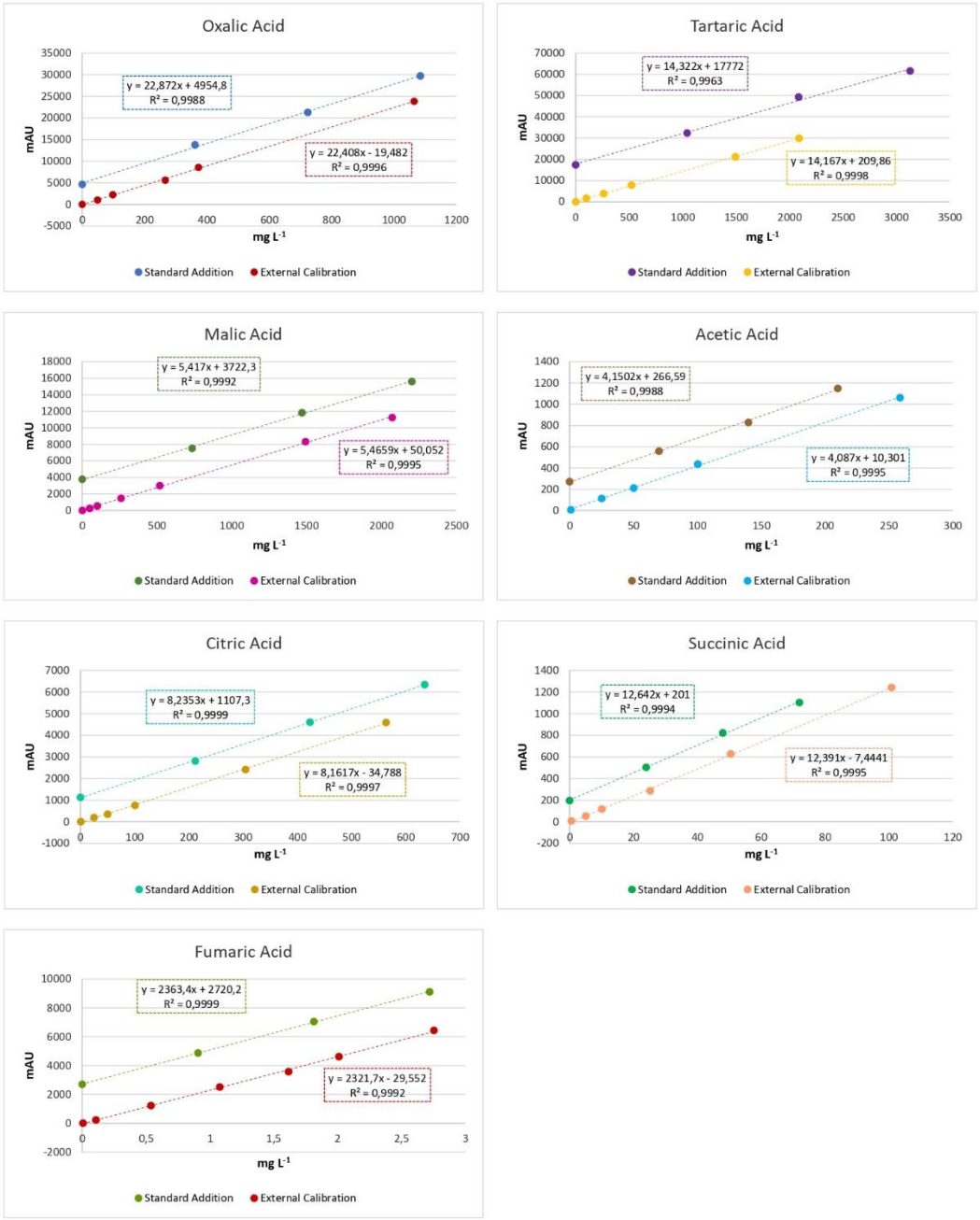

Figure IV.2. *Comparison of the matrix effect for Oxalic acid (a), Tartaric acid (b), Malic acid (c), Acetic acid (d), Citric acid (e), Succinic acid (f), and Fumaric acid (g) in* Vitis vinifera L. *musts.*

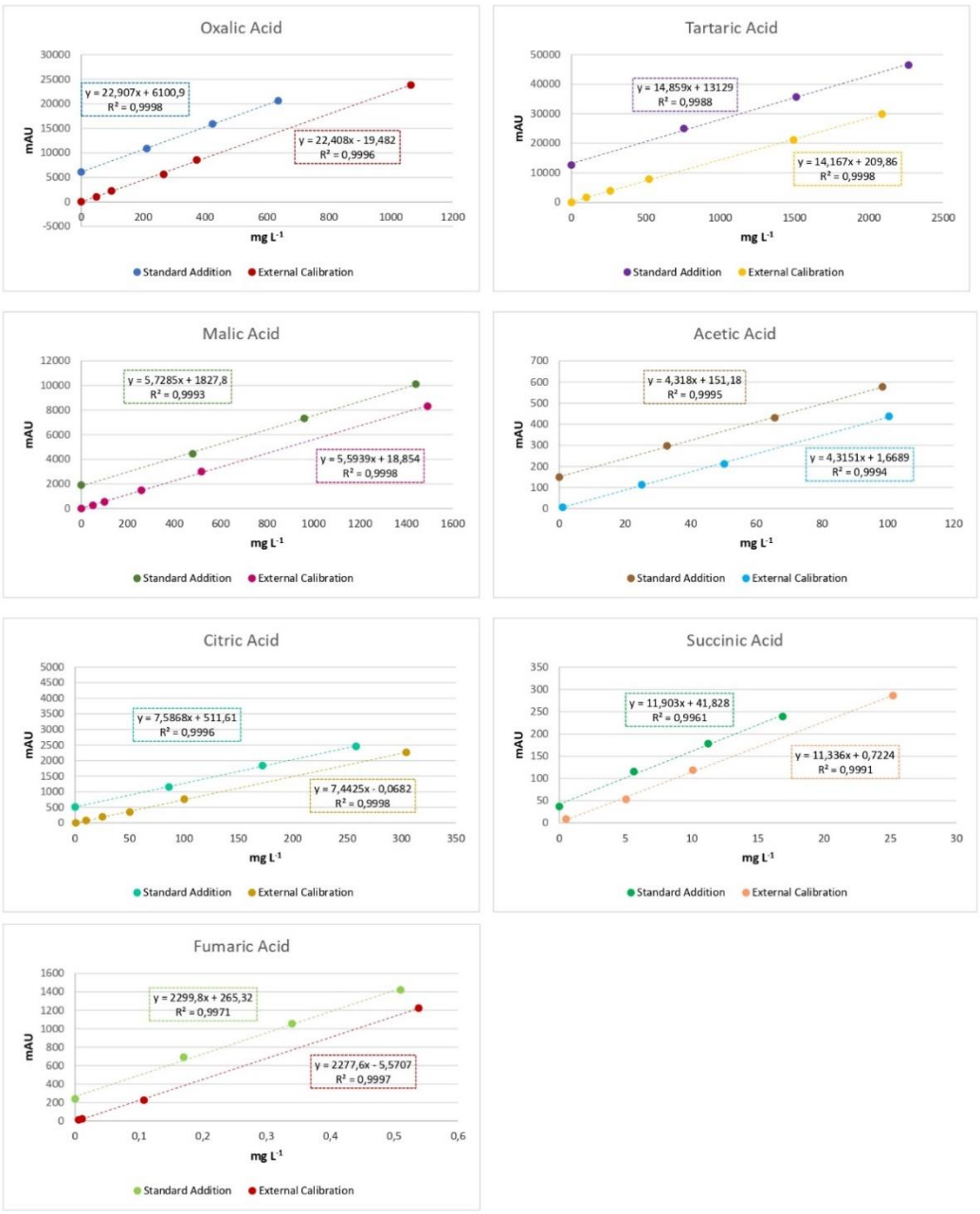

Figure IV.3. *Comparison of the matrix effect for Oxalic acid (a), Tartaric acid (b), Malic acid (c), Acetic acid (d), Citric acid (e), Succinic acid (f) and Fumaric acid (g) in* Vitis vinifera *L. grapes.*

ANNEX V: STUDY OF THE MATRIX EFFECT IN THE QUANTIFICATION OF AMINO ACIDS USING HPLC-FLD.

Table V.1. *Standard addition and external calibration curves and matrix coefficient in a pooled leaf of* Vitis vinifera *L. for amino acids quantification.*

	Slope S.A. ± error	Slope E.C. ± error	Matrix Effect (%)
Asp	5.1 ± 0.1	4.9 ± 0.1	-3.5
Glu	6.6 ± 0.2	6.4 ± 0.1	-1.9
Asn	7.6 ± 0.2	7.4 ± 0.1	-2.3
Gln	3.66 ± 0.1	3.5 ± 0.0	-2.5
Arg	9.8 ± 0.2	9.9 ± 0.1	1.7
Ala	11.8 ± 0.4	11.4 ± 0.1	-3.3
GABA	13.0 ± 0.3	12.4 ± 0.1	-4.2
BABA	12.9 ± 0.3	13.4 ± 0.2	3.8

S.A. Standard Addition method. E.C. External Calibration method.

Table V.2. *Standard addition and external calibration curves and matrix coefficient in a pooled must of* Vitis vinifera *L. for amino acids quantification.*

	Slope S.A. ± error	Slope E.C. ± error	Matrix Effect (%)
Asp	2.8 ± 0.0	2.9 ± 0.1	4.8
Glu	3.0 ± 0.1	3.0 ± 0.0	-0.2
Asn	3.9 ± 0.1	3.9 ± 0.0	0.1
Gln	4.5 ± 0.0	4.6 ± 0.1	2.8
Arg	3.5 ± 0.1	3.6 ± 0.0	4.0
Ala	6.8 ± 0.2	7.0 ± 0.1	2.3
GABA	5.6 ± 0.0	5.7 ± 0.1	1.1
BABA	5.0 ± 0.1	4.9 ± 0.0	-0.8

S.A. Standard Addition method. E.C. External Calibration method.

Table V.3. *Standard addition and external calibration curves and matrix coefficient in a pooled grape of* Vitis vinifera *L. for amino acids quantification.*

	Slope S.A. ± error	Slope E.C. ± error	Matrix Effect (%)
Asp	1.9 ± 0.0	1.9 ± 0.0	-0.1
Glu	1.9 ± 0.0	1.9 ± 0.0	-1.2
Asn	2.4 ± 0.1	2.4 ± 0.0	0.8
Gln	2.7 ± 0.1	2.8 ± 0.0	4.0
Arg	2.3 ± 0.0	2.4 ± 0.0	1.5
Ala	4.3 ± 0.1	4.3 ± 0.0	-0.1
GABA	3.8 ± 0.1	3.8 ± 0.0	0.1
BABA	2.3 ± 0.0	2.4 ± 0.0	-0.4

S.A. Standard Addition method. E.C. External Calibration method.

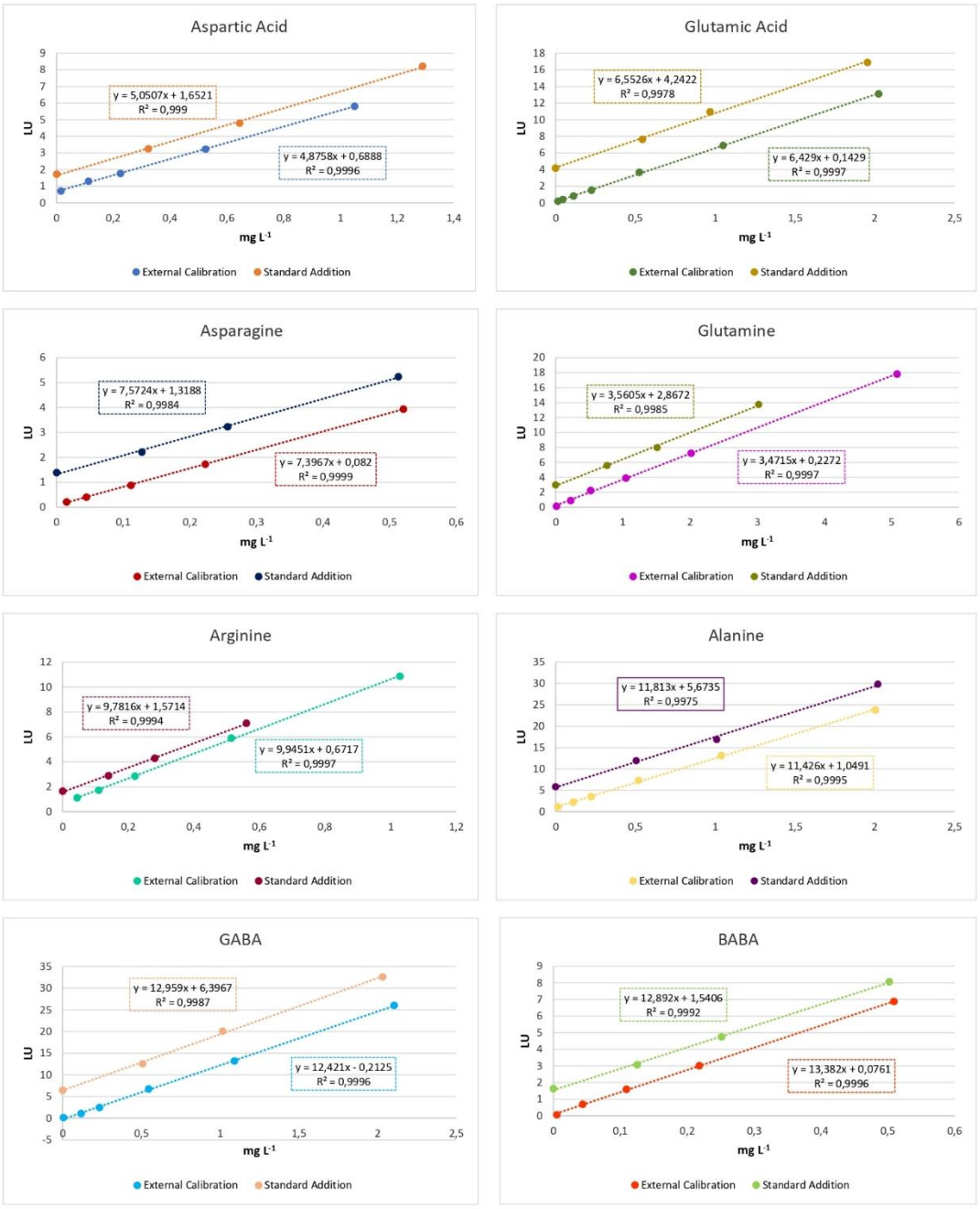

Figure V.1. *Matrix effect for Aspartic (a), Glutamic (b), Asparagine (c), Glutamine (d), Arginine (e), Alanine (f), GABA (g) and BABA (h) in a pooled leaf of* Vitis vinifera *L.*

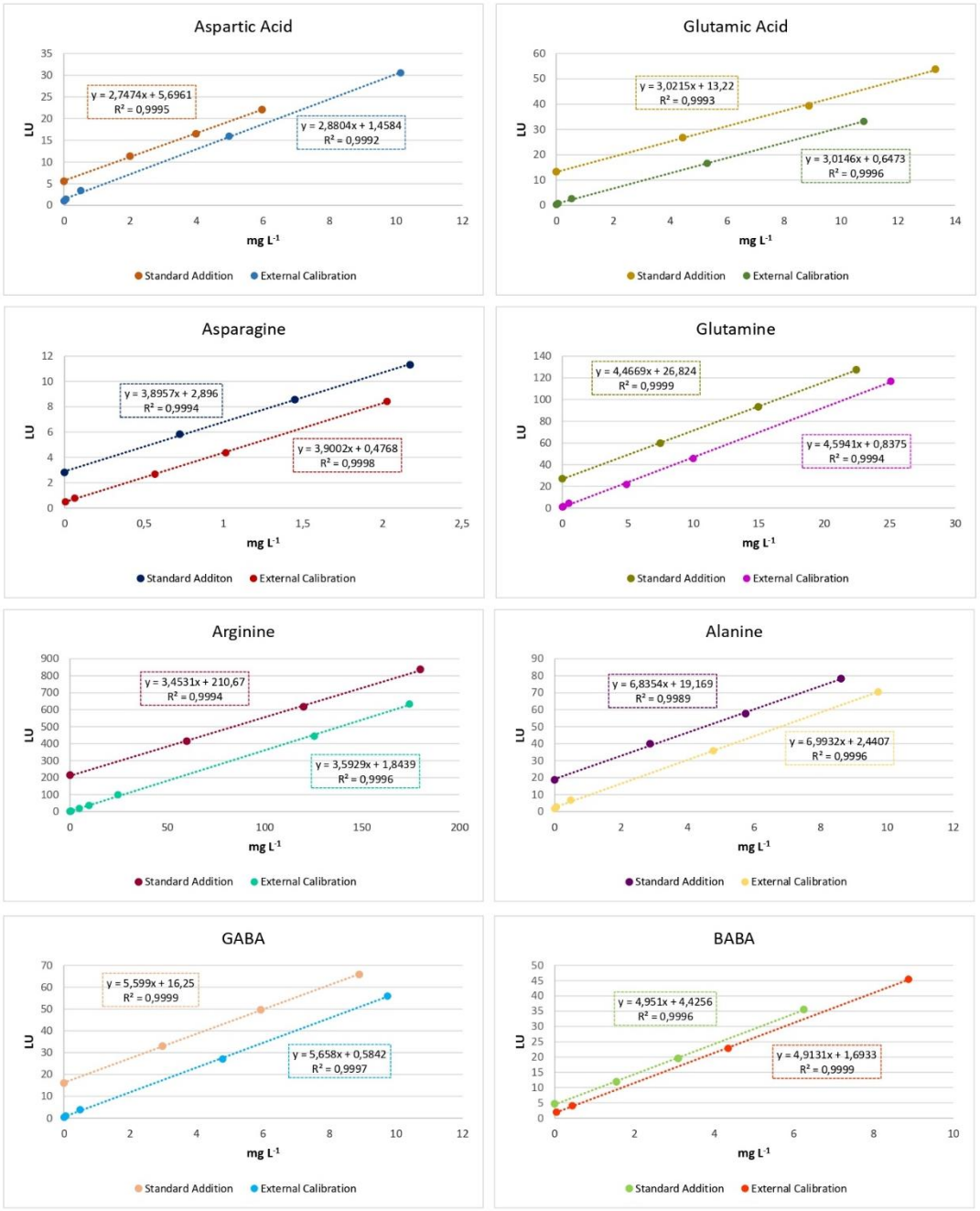

Figure V.2. *Matrix effect for Aspartic (a), Glutamic (b), Asparagine (c), Glutamine (d), Arginine (e), Alanine (f), GABA (g) and BABA (h) in a pooled must of* Vitis vinifera *L.*

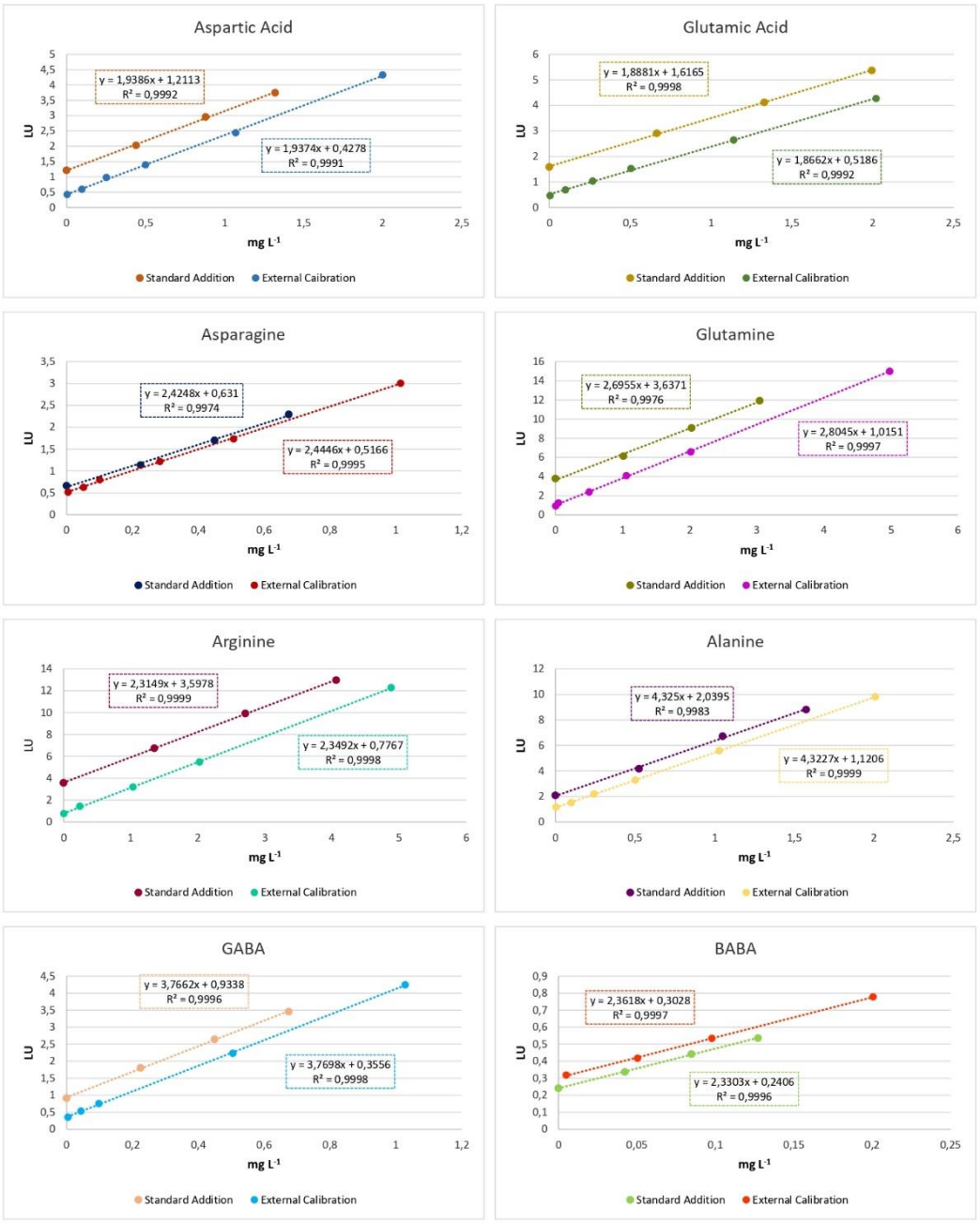

Figure V.3. *Matrix effect for Aspartic (a), Glutamic (b), Asparagine (c), Glutamine (d), Arginine (e), Alanine (f), GABA (g) and BABA (h) in a pooled grape sample of* Vitis vinifera *L.*

ANNEX VI: DESCRIPTION OF FLUORCAM PARAMETERS.

Table VI.1. *Chlorophyll fluorescence related parameters measured by the FluorCam protocol*[369].

Símbolo	Fórmula	Nombre	Descripción
F_o	Measured	minimum fluorescence in dark-adapted state QA oxidized (qP=1). non-photochemical quenching relaxed (NPQ=0)	minimum fluorescence in dark-adapted state QA oxidized (qP=1). non-photochemical quenching relaxed (NPQ=0)
F_o_Dn	Measured	minimum fluorescence during dark relaxation QA oxidized (qP=1). non-photochemical quenching relaxing (NPQ>0)	minimum fluorescence during dark relaxation QA oxidized (qP=1). non-photochemical quenching relaxing (NPQ>0)
F_o_Ln	F_o/[(F_m- F_o)/(F_m+ F_o/ F_m_Ln)]	minimum fluorescence during light adaptation Calculated estimate: QA oxidized (qP=1). non-photochemical quenching induced (NPQ>0)	minimum fluorescence during light adaptation Calculated estimate: QA oxidized (qP=1). non-photochemical quenching induced (NPQ>0)
F_o_Lss	Measured	steady-state minimum fluorescence in light QA oxidized (qP=1). non-photochemical quenching at maximum (NPQ max)	steady-state minimum fluorescence in light QA oxidized (qP=1). non-photochemical quenching at maximum (NPQ max)
F_m	Measured	maximum fluorescence in dark-adapted state QA reduced (qP=0). non-photochemical quenching relaxed (NPQ=0	maximum fluorescence in dark-adapted state QA reduced (qP=0). non-photochemical quenching relaxed (NPQ=0
F_m_Dn	Measured	instantaneous maximum fluorescence during dark relaxation QA reduced (qP=0). non-photochemical quenching relaxing (NPQ>0)	instantaneous maximum fluorescence during dark relaxation QA reduced (qP=0). non-photochemical quenching relaxing (NPQ>0)
F_m_Ln	Measured	maximum fluorescence during light adaptation QA reduced (qP=0). non-photochemical quenching induced (NPQ>0)	maximum fluorescence during light adaptation QA reduced (qP=0). non-photochemical quenching induced (NPQ>0)
F_m_Lss	Measured	steady-state maximum fluorescence in light QA reduced (qP=0). non-photochemical quenching at maximum (NPQ max)	steady-state maximum fluorescence in light QA reduced (qP=0). non-photochemical quenching at maximum (NPQ max)
F_p	Measured	peak fluorescence during the initial phase of the Kautsky effect local F-maximum resulting from rapid reduction of plastoquinone pool and slower activation of re-oxidation mechanisms and of non-photochemical quenching	peak fluorescence during the initial phase of the Kautsky effect local F-maximum resulting from rapid reduction of plastoquinone pool and slower activation of re-oxidation mechanisms and of non-photochemical quenching

Table VI.1(cont.). *Chlorophyll fluorescence related parameters measured by the FluorCam protocol[369].*

Símbolo	Fórmula	Nombre	Descripción
F_t_Dn	Measured	instantaneous fluorescence during dark relaxation instantaneous F-level during dark relaxation that results from a dynamic equilibrium of plastoquinone reducing and re-oxidizing processes and from non-photochemical quenching	instantaneous fluorescence during dark relaxation instantaneous F-level during dark relaxation that results from a dynamic equilibrium of plastoquinone reducing and re-oxidizing processes and from non-photochemical quenching
F_t_Ln	Measured	instantaneous fluorescence during light adaptation instantaneous F-level during light adaptation that results from a dynamic equilibrium of plastoquinone reducing and re-oxidizing processes and from non-photochemical quenching	instantaneous fluorescence during light adaptation instantaneous F-level during light adaptation that results from a dynamic equilibrium of plastoquinone reducing and re-oxidizing processes and from non-photochemical quenching
F_t_Lss	Measured	steady-state fluorescence in light steady-state F-level that results from a dynamic equilibrium of plastoquinone reducing and re-oxidizing processes and from non-photochemical quenching	steady-state fluorescence in light steady-state F-level that results from a dynamic equilibrium of plastoquinone reducing and re-oxidizing processes and from non-photochemical quenching
F_v	F_m - F_o	variable fluorescence in dark-adapted state Variable fluorescence increment that is due the transition from dark-adapted state with all-open reaction centers to the all-closed state during saturating flash of light	variable fluorescence in dark-adapted state Variable fluorescence increment that is due the transition from dark-adapted state with all-open reaction centers to the all-closed state during saturating flash of light
NPQ_Dn	(F_m- F_m_Dn)/ F_m_Dn	nstantaneous non-photochemical quenching during dark relaxation non-photochemical quenching relaxing in dark	nstantaneous non-photochemical quenching during dark relaxation non-photochemical quenching relaxing in dark
NPQ_Ln	(Fm- Fm_Ln)/ Fm_Ln	instantaneous non-photochemical quenching during light adaptation non-photochemical quenching induced in light	instantaneous non-photochemical quenching during light adaptation non-photochemical quenching induced in light
NPQ_Lss	(Fm- Fm_Lss)/ Fm_Lss	steady-state non-photochemical quenching	steady-state non-photochemical quenching in light
qP_Dn	(Fm_Dn- Ft_Dn)/(Fm_Dn-Fo_Dn)	coefficient of photochemical quenching during dark relaxation estimates of the fraction of open PSII reaction centers PSIIopen / (PSIIopen+ PSIIclosed)	coefficient of photochemical quenching during dark relaxation estimates of the fraction of open PSII reaction centers PSIIopen / (PSIIopen+ PSIIclosed)
qP_Ln	(F_m_Ln- F_t_Ln)/(F_m_Ln-F_o_Ln)	during light adaptation estimate of the fraction of open PSII reaction centers PSIIopen / (PSIIopen+ PSIIclosed)	during light adaptation estimate of the fraction of open PSII reaction centers PSIIopen / (PSIIopen+ PSIIclosed)

Table VI.1(cont.). *Chlorophyll fluorescence related parameters measured by the FluorCam protocol*[369].

Símbolo	Fórmula	Nombre	Descripción
qP_Lss	F_m_Lss- F_t_Lss)/(F_m_Lss- F_o_Lss)	coefficient of photochemical quenching in steady-state estimate of the fraction of open PSII reaction centers PSIIopen / (PSIIopen+ PSIIclosed)	coefficient of photochemical quenching in steady-state estimate of the fraction of open PSII reaction centers PSIIopen / (PSIIopen+ PSIIclosed)
QY_Dn	(F_m_Dn- F_t_Dn)/ F_m_Dn	instantaneous PSII quantum yield during dark relaxation PSII quantum yield relaxing in dark	instantaneous PSII quantum yield during dark relaxation PSII quantum yield relaxing in dark
QY_Ln	(F_m_Ln- F_t_Ln)/ F_m_Ln	instantaneous PSII quantum yield during light adaptation	PSII quantum yield induced in light
QY_Lss	(F_m_Lss- F_t_Lss)/ F_m_Lss	steady-state PSII quantum yield steady-state PSII quantum yield in light	steady-state PSII quantum yield steady-state PSII quantum yield in light
F_v/F_m (QY_max)	F_v/F_m	maximum PSII quantum yield maximum PSII quantum yield in dark-adapted stat	maximum PSII quantum yield maximum PSII quantum yield in dark-adapted state
F_v/F_m_Ln	(F_m_Ln- F_o_Lss)/ F_m_Ln	PSII quantum yield of light adapted sample PSII quantum yield in light-adapted state	PSII quantum yield of light adapted sample PSII quantum yield in light-adapted state
F_v/F_m_Lss	(F_m_Lss- F_o_Lss)/ F_m_Lss	PSII quantum yield of light adapted sample at steady-state PSII quantum yield in light-adapted steady-state	PSII quantum yield of light adapted sample at steady-state PSII quantum yield in light-adapted steady-state
R_{fd}_Ln	(F_p- F_t_Ln)/ F_t_Ln	instantaneous fluorescence decline ratio in light empiric parameter used to assess plant vitality	instantaneous fluorescence decline ratio in light empiric parameter used to assess plant vitality
qP_Ln	(F_m_Ln- F_t_Ln)/(F_m_Ln- F_o_Ln)	during light adaptation estimate of the fraction of open PSII reaction centers PSIIopen / (PSIIopen+ PSIIclosed)	during light adaptation estimate of the fraction of open PSII reaction centers PSIIopen / (PSIIopen+ PSIIclosed)

ANNEX VII: DESCRIPTION OF OPTIMAL MRM TRANSITIONS FOR BEH AMIDE COLUMNS.

Table VII.1. *Retention times and optimal MRM conditions of analytes and internal Standards separated on BEH Amide column.*

Compound	Rt (min) 50 × 2.1 mm	MRM transitions (Collision energy V)	Rel. intensity (%)
TyrP	1.116±0.020 (1.81)	160.95>144.10 (14)	100
		160.95>115.10 (34)	46
		160.95>116.95 (24)	41
TyrA	1.502±0.030 (2.02)	137.90>121.05 (14)	100
		137.90>77.00 (28)	85
		137.90>91.05 (23)	36
Leu	2.945±0.058 (1.97)	132.10>86.05 (12)	100
		132.10>44.15 (23)	20
Leu IS	2.957±0.061 (2.07)	141.95>96.10 (12)	100
Ile	3.275±0.062 (1.90)	132.10>86.15 (12)	100
		132.10>68.95 (16)	20
Phe	3.030±0.058 (1.92)	165.90>120.10 (15)	100
		65.90>103.10 (27)	58
		165.90>77.00 (40)	56
Trp	3.335±0.062 (1.86)	205.10>188.20 (11)	100
		205.10>118.00 (25)	81
		205.10>146.00 (18)	71
BABA	3.370±0.058 (2.03)	104.10>45.05 (24)	100
		104.10>41.00 (30)	57
GABA	3.372±0.083 (2.45)	104.10>87.10 (14)	100
		104.10>45.05 (23)	60
		104.10>69.00 (16)	37

Table VII.1 (cont.). *Retention times and optimal MRM conditions of analytes and internal Standards separated on BEH Amide column.*

Compound	Rt (min) 50 × 2.1 mm	MRM transitions (Collision energy V)	Rel. intensity (%)
Met	3.838±0.054 (1.40)	149.90>56.10 (17)	100
		149.90>61.00 (23)	85
		149.90>104.00 (13)	56
Val	4.126±0.066 (1.60)	118.00>72.00 (10)	100
		118.00>55.05 (20)	42
Pro IS	4.149±0.064 (1.55)	122.10>75.00 (16)	100
		122.10>46.10 (30)	3
		122.10>30.10 (38)	3
Pro	4.157±0.069 (1.66)	116.10>70.05 (7)	100
		116.10>43.05 (22)	4
β-Ala	4.623±0.105 (2.27)	90.10>72.10 (12)	100
		90.10>30.10 (13)	97
		90.10>45.15 (33)	33
Cys	4.590±0.082 (1.78)	121.90>38.85 (14)	100
		121.90>81.00 (7)	42
Tyr	4.703±0.051 (1.09)	182.10>91.10 (28)	100
		182.10>136.00 (15)	48
		181.10>165.15 (13)	35
Ala	5.460±0.059 (1.08)	90.10>44.10 (12)	100
		90.10>45.00 (31)	8
Hpr	5.501±0.025 (0.45)	132.90>87.10 (15)	100
		132.90>69.00 (22)	86
		132.90>68.05 (22)	23

Table VII.1 (cont.). *Retention times and optimal MRM conditions of analytes and internal Standards separated on BEH Amide column.*

Compound	Rt (min) 50 × 2.1 mm	MRM transitions (Collision energy V)	Rel. intensity (%)
Thr	5.706±0.055 (0.97)	120.10>74.00 (14)	100
		120.10>102.10 (15)	22
AAA	5.776±0.021 (0.36)	161.90>98.00 (15)	100
		161.90>55.10 (26)	53
		161.90>121.00 (10)	9
Thr IS	5.703±0.021 (0.37)	129.90>80.00 (13)	100
		129.90>65.10 (18)	24
Gly	5.794±0.027 (0.47)	76.10>30.00 (11)	100
		76.10>48.00 (14)	3
Glu IS	5.965±0.017 (0.28)	152.95>88.00 (18)	100
		152.95>89.20 (16)	60
		152.95>135.10 (14)	28
Glu	5.978±0.035 (0.58)	147.90>84.10 (17)	100
		147.90>56.00 (20)	17
Gly	5.794±0.027 (0.47)	76.10>30.00 (11)	100
		76.10>48.00 (14)	3
Glu IS	5.965±0.017 (0.28)	152.95>88.00 (18)	100
		152.95>89.20 (16)	60
		152.95>135.10 (14)	28
Glu	5.978±0.035 (0.58)	147.90>84.10 (17)	100
		147.90>56.00 (20)	17

Table VII.1 (cont.). *Retention times and optimal MRM conditions of analytes and internal Standards separated on BEH Amide column.*

Compound	Rt (min) 50 × 2.1 mm	MRM transitions (Collision energy V)	Rel. intensity (%)
Gln IS	6.065±0.012 (0.20)	151.90>88.00 (18)	100
		151.90>135.10 (14)	88
		151.90>89.20 (19)	72
Gln	6.065±0.041 (0.68)	146.90>84.10 (18)	100
		146.90>130.10 (15)	37
		146.90>56.05 (29)	25
Ser	6.096±0.077 (1.26)	106.00>60.00 (15)	100
		106.10>88.00 (11)	14
Asn	6.156±0.020 (0.32)	132.90>74.00 (16)	100
		132.90>87.15 (11)	53
		132.90>88.10 (12)	11
Asp	6.287±0.072 (1.14)	134.00>88.00 (10)	100
		134.00>74.00 (12)	82
Cit	6.296±0.008 (0.120	175.90>70.05 (23)	100
		175.90>159.20 (14)	39
		175.90>113.05 (17)	24
Arg	6.850±0.009 (0.12)	175.00>70.05 (23)	100
		175.00>116.10 (15)	67
His	6.847±0.019 (0.27)	155.90>110.15 (16)	100
		155.90>83.10 (24)	45
Lys	6.927±0.012 (0.18)	146.90>84.10 (18)	100
		146.90>130.00 (15	20
		; 146.90>56.15 (28)	18

Table VII.1 (cont.). *Retention times and optimal MRM conditions of analytes and internal Standards separated on BEH Amide column.*

Compound	Rt (min) 50 × 2.1 mm	MRM transitions (Collision energy V)	Rel. intensity (%)
Orn	6.967±0.014 (0.20)	133.10>70.00 (22)	100
		133.10>116.00 (15)	84
		133.10>43.15 (33)	59
Cis	7.185±0.013 (0.18)	241.00>74.00 (29)	100
		214.00>151.95 (14)	62

ANNEX VIII: DATA USED IN THE STATISTICAL ANALYSIS OF *VITIS VINIFERA* L.

Table VIII.1. *Content of carbohydrates (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *leaves after first and 24 hours before first application (T0A and T24B), after and 24 hours before the second foliar application (T0B and T24B) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot A and B.*

		Fruc		Gluc		Sac		Malt	
		A	**B**	**A**	**B**	**A**	**B**	**A**	**B**
C	**T0A**	4,703.5±666.2	1,071.7±155.7	6,208.5±744.0	1,388.4±203.7	<LOD	<LOD	6,538.9±803.6	1,478.2±171.2
	T24A	1,909.8±119.5	1,139±62.8	2,608.6±209.1	1,204.7±95.9	<LOD	<LOD	1,700.6±147.5	455.6±28.3
	T0B	2,119.9±255.5	1,405.4±174.6	3,382.4±361.9	2,608.1±348.3	<LOD	<LOD	2,015.1±249.3	1,788.6±194.5
	T24B	1,917.1±18.8	1,374±20.8	3925.2±50.3	2,011.1±24.6	<LOD	<LOD	2,5617.1±597.5	2,017.9±43
	TF	1,218.9±78.3	1,602.3±129.7	1,991.8±126.9	1,842.3±140.8	<LOD	<LOD	694.7±44.2	1,453.5±76.4
BABA	**T0A**	3,038.8±442.2	693.2±130.1	5,430.7±826	180.5±31.0	<LOD	<LOD	5,094.6±721.2	1,390.5±243.3
	T24A	1,569.5±118.6	750.1±42.3	2,141.2±147.8	962.0±57.6	<LOD	<LOD	1,788.5±159.5	<LOD
	T0B	1,799.8±2.7	1,332.4±48.0	2,534.7±1.2	1,509.6±21.7	<LOD	<LOD	2,080.7±10.0	1,249.1±0.2
	T24B	2,037.4±162.0	1,340.8±99.3	2,621.1±154.6	1,718.1±112.4	<LOD	<LOD	2,665.3±228.4	2,265.6±137
	TF	2,386.1±22.4	2,470.7±138.7	2,857.2±37.5	3,028.2±40.8	<LOD	<LOD	<LOD	2,401.2±116.7
GT	**T0A**	1,414.5±7.2	1,919.3±6.7	1,907.7±8.6	2,030.3±87.7	<LOD	<LOD	1,582.2±1.9	1,343.6±28.6
	T24A	2,493.9±58.0	1,694.4±23.6	3,408.1±82.9	1,961±44.7	<LOD	<LOD	1,987.6±46.1	1,241.1±1.6
	T0B	1,207.7±47.3	1,657±84.7	2,564.2±98.6	2,371.3±113.9	<LOD	<LOD	1,287.4±56.3	1,270.1±39.4
	T24B	1,221±74.1	1,260.5±97.2	1,926.8±125.3	2,142.7±179.1	<LOD	<LOD	1,819.8±63.7	2,099.6±34.6
	TF	2,330.2±253.9	1,689.1±141.9	2,591.9±228.1	1,628.7±173.9	<LOD	<LOD	2,012.3±200	1,496.4±164.3
BF	**T0A**	1,369.0±75.0	1,197.1±90.5	1,926.3±141.3	1,110.4±63.3	<LOD	<LOD	1,258.2±72.8	1,759.7±147.7
	T24A	1,144.8±16.7	852.1±24.9	1,571.9±13.6	959.6±10.8	<LOD	<LOD	959.8±17	359.6±8.5
	T0B	1,950.3±164.0	1,936.2±120.8	2,705.3±225.3	2,468±158.2	<LOD	<LOD	2,085±175.2	2,154.2±263.6
	T24B	2,323.4±122.6	1,487.7±11.0	4,540.2±136.3	2,333.2±25.9	<LOD	<LOD	3,252.3±175.3	4,141±26.1
	TF	4,317.1±233.2	2,010.6±131.4	1,541.3±38.7	1,803.8±31.7	<LOD	<LOD	<LOD	<LOD
ST	**T0A**	1,027±483.8	1,163.0±548.0	1,497.5±704.2	1,702.2±804.8	<LOD	<LOD	1,151.3±541.7	1,381.5±650.8
	T24A	1,627.9±114.6	1,180.3±87.5	2,235.8±218.9	1,571.2±115.8	<LOD	<LOD	1,214.6±126.5	<LOD
	T0B	5,626.8±639.8	1,676.4±202.1	3,511.9±515.4	2,111.9±291.2	<LOD	<LOD	7,840.3±1174.1	1,384.8±184.3
	T24B	2,577.3±0.5	1,762±3.8	2,739.0±19.6	3,511.3±7.6	<LOD	<LOD	2,789.7±64.2	2447.5±50.9
	TF	791.6±56.6	1,482.4±85.4	1,270.5±107.7	1,902.1±164.0	<LOD	<LOD	<LOD	1,080.7±62.5

Table VIII.2. *Content of carbohydrates (µmol L^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *musts at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot A and B.*

		Fruc		Gluc	
		A	**B**	**A**	**B**
C	**TV**	884,360.5±9,993.7	<LOD	1,049,309.5±2,503.0	<LOD
	TF	724,904.2±7,05.7	606,707.4±10,221.7	785,713.5±442.6	679,600.7±11,414.6
BABA	**TV**	615,891.6±2,557.6	756,290.9±1,857.9	749,092.0±11,664.3	825,220.2±4,202.3
	TF	718,478.8±3,720.5	724,163.9±15,919.4	755,874.4±9,957.6	793,131.7±16,608.5
GT	**TV**	732,061.7±6,607.6	745,104.8±11,179.3	794,520.3±15,061.8	762,302.8±6,511.2
	TF	578,185.0±11,076.6	594,073.7±9,795.5	623,712.3±1,476.3	756,066.4±5,369.2
BF	**TV**	54,0511.5±4,167.1	749,768.3±5,404.0	608,671.8±3,801.7	860,777.7±5,333.9
	TF	732,415.3±1,775.5	643,195.9±3,081.7	838,741.8±16,295.8	929,498.8±14,248.8
ST	**TV**	654,998.2±2,562.3	677,525.4±14,393.5	777,844.3±4,841.6	702,380.5±9,398.0
	TF	403,278.2±5,183.3	600,709.3±13,166.6	460,223.5±4,233.5	749,637.6±8,434.9

Table VIII.3. *Content of carbohydrates (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *grapes at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot A and B.*

		Fruc		Gluc	
		A	**B**	**A**	**B**
C	**TV**	298.5±2.5	318.7±3.3	316.6±1.5	409.4±4.8
	TF	357.0±0.1	327.8±3.8	404.2±10.5	412.6±5.0
BABA	**TV**	301.2±0.4	434.8±8.3	310.0±6.3	519.6±8.1
	TF	335.0±0.4	474.9±8.6	426.4±2.2	575.9±6.1
GT	**TV**	338.2±4.5	451.2±3.2	407.3±2.8	526.6±3.5
	TF	323.4±2.3	294.4±2.1	394.3±3.3	371.3±4.4
BF	**TV**	292.0±0.2	287.7±2.3	344.0±2.3	312.0±2.7
	TF	315.4±2.4	266.1±1.5	423.5±2.5	324.7±6.2
ST	**TV**	225.4±1.1	343.0±4.0	272.1±2.3	405.0±4.6
	TF	411.9±5.1	<LOD	471.5±4.4	<LOD

Table VIII.4A. *Content of organic acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *leaves after first and 24 hours before first application (T0A and T24B), after and 24 hours before the second foliar application (T0B and T24B) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot A.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
C	T0A	0.0±0.0	1.5±0.0	2.4±0.0	12.9±0.2	20.1±0.0	23.0±0.1	10.9±0.0
	T24A	0.0±0.0	1.2±0.0	2.5±0.0	15.9±0.1	17.2±0.0	25.4±0.0	14.2±0.1
	T0B	0.0±0.0	1.2±0.0	1.5±0.0	6.2±0.0	17.6±0.0	19.5±0.0	11.7±0.1
	T24B	0.0±0.0	3.0±0.0	3.3±0.0	13.7±0.0	17.2±0.0	18.5±0.0	18.0±0.1
	TF	0.0±0.0	2.0±0.0	0.7±0.0	22.0±0.1	30.7±0.0	37.2±0.2	10.6±0.0
BABA	T0A	0.0±0.0	0.9±0.0	0.6±0.0	5.2±0.1	22.2±0.0	21.7±0.0	18.1±0.1
	T24A	0.0±0.0	0.6±0.0	1.0±0.0	8.3±0.0	11.9±0.0	16.0±0.1	12.3±0.0
	T0B	0.0±0.0	0.9±0.0	0.5±0.0	3.5±0.1	16.4±0.1	18.1±0.0	17.2±0.1
	T24B	0.0±0.0	2.0±0.0	0.9±0.0	9.8±0.0	17.1±0.0	15.3±0.1	16.2±0.2
	TF	0.0±0.0	1.0±0.0	0.4±0.0	5.0±0.0	15.5±0.1	20.7±0.0	7.6±0.1
GT	T0A	0.0±0.0	0.7±0.0	1.0±0.0	9.1±0.0	14.0±0.0	19.3±0.0	12.7±0.0
	T24A	0.0±0.0	1.0±0.0	0.9±0.0	10.5±0.1	21.0±0.0	23.1±0.1	16.2±0.0
	T0B	0.0±0.0	8.0±0.0	0.7±0.0	7.3±0.1	14.7±0.0	14.7±0.0	13.9±0.1
	T24B	0.0±0.0	0.9±0.0	1.2±0.0	11.2±0.0	19.3±0.0	23.3±0.0	21.8±0.0
	TF	0.0±0.0	1.4±0.0	1.0±0.0	16.2±0.0	23.5±0.3	32.4±0.2	15.5±0.1
BF	T0A	0.0±0.0	0.9±0.0	1.0±0.0	7.6±0.1	13.4±0.0	17.9±0.0	11.8±0.2
	T24A	0.0±0.0	0.7±0.0	1.0±0.0	5.0±0.0	14.4±0.0	16.0±0.0	12.0±0.1
	T0B	0.0±0.0	0.7±0.0	0.6±0.0	5.7±0.0	12.5±0.0	16.7±0.1	10.2±0.1
	T24B	0.0±0.0	8.0±0.0	0.9±0.0	5.5±0.0	12.1±0.0	13.6±0.1	11.7±0.0
	TF	0.0±0.0	0.7±0.0	8.0±0.0	6.3±0.1	9.3±0.0	18.3±0.0	7.4±0.0
ST	T0A	0.0±0.0	1.0±0.0	1.3±0.0	15.9±0.1	15.6±0.1	25.7±0.1	20.7±0.2
	T24A	0.0±0.0	0.6±0.0	1.0±0.0	8.0±0.1	13.6±0.0	18.1±0.0	18.5±0.2
	T0B	0.0±0.0	0.6±0.0	8.0±0.0	7.9±0.0	14.9±0.0	20.0±0.1	15.1±0.1
	T24B	0.0±0.0	0.5±0.0	0.3±0.0	5.9±0.0	14.2±0.0	16.1±0.0	15.2±0.1
	TF	0.0±0.0	1.0±0.0	0.3±0.0	25.2±0.5	16.9±0.2	40.4±0.2	10.1±0.2

Table VIII.4B. *Content of organic acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *leaves after first and 24 hours before first application (T0A and T24B), after and 24 hours before the second foliar application (T0B and T24B) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot B.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
C	T0A	0.0±0.0	1.4±0.0	8.0±0.0	63.3±0.1	20.7±0.0	31.0±0.0	14.5±0.0
	T24A	0.0±0.0	8.0±0.0	0.3±0.0	54.1±0.5	10.3±0.0	26.1±0.1	6.9±0.0
	T0B	0.0±0.0	1.5±0.0	0.6±0.0	27.9±0.0	18.6±0.0	22.8±0.0	10.4±0.0
	T24B	0.0±0.0	0.4±0.0	0.9±0.0	3.0±0.0	16.9±0.0	7.0±0.0	3.8±0.0
	TF	0.0±0.0	0.5±0.0	1.4±0.1	3.6±0.0	29.4±0.1	6.8±0.1	5.7±0.1
BABA	T0A	0.0±0.0	1.6±0.0	0.7±0.0	16.1±0.3	14.3±0.1	20.6±0.0	13.5±0.1
	T24A	0.0±0.0	1.7±0.0	0.6±0.0	13.0±0.1	25.3±0.1	26.5±0.1	13.1±0.1
	T0B	0.0±0.0	2.3±0.0	0.6±0.0	13.1±0.3	13.0±0.0	19.3±0.0	10.6±0.0
	T24B	0.0±0.0	8.0±0.0	1.6±0.0	5.0±0.1	26.3±0.1	7.2±0.0	6.3.0±0.0
	TF	0.0±0.0	0.6±0.0	1.7±0.0	5.0±0.2	21.8±0.0	13.2±0.0	11.5±0.0
GT	T0A	0.0±0.0	0.9±0.0	1.4±0.0	10.5±0.0	32.3±0.0	28.1±0.0	14.2±0.1
	T24A	0.0±0.0	0.7±0.0	8.0±0.0	12.7±0.1	16.4±0.0	21.3±0.1	11.8±0.0
	T0B	0.0±0.0	0.9±0.0	1.3±0.0	9.7±0.1	28.4±0.1	22.9±0.1	15.8±0.0
	T24B	0.0±0.0	1.8±0.0	2.5±0.0	8.8±0.0	33.7±0.0	17.9±0.1	21.2±0.0
	TF	0.0±0.0	1.2±0.0	3.4±0.0	6.8±0.0	47.8±0.1	10.3±0.0	23.3±0.1
BF	T0A	0.0±0.0	3.0±0.0	0.9±0.0	11.4±0.1	21.8±0.0	17.9±0.0	16.3±0.0
	T24A	0.0±0.0	1.4±0.0	1.2±0.0	10.3±0.2	23.6±0.1	31.4±0.0	10.7±0.1
	T0B	0.0±0.0	1.3±0.0	0.9±0.0	7.4±0.0	12.0±0.1	18.5±0.0	10.9±0.0
	T24B	0.0±0.0	1.7±0.0	1.8±0.1	4.5±0.1	15.9±0.0	16.3±0.1	8.7±0.1
	TF	0.0±0.0	1.2±0.0	1.7±0.0	3.9±0.0	20.5±0.1	7.8.0±0.1	6.0±0.0
ST	T0A	0.0±0.0	1.0±0.0	0.9±0.0	9.3±0.1	33.9±0.0	24.5±0.2	12.0±0.1
	T24A	0.0±0.0	1.2±0.0	0.3±0.0	15.5±0.2	15.2±0.0	27.2±0.2	7.1±0.2
	T0B	0.0±0.0	1.3±0.0	0.5±0.0	9.3±0.1	18.1±0.0	19.8±0.1	8.3±0.0
	T24B	0.0±0.0	0.6±0.0	0.9±0.0	2.2±0.0	22.7±0.1	6.7±0.0	6.5±0.0
	TF	0.0±0.0	0.6±0.0	0.6±0.0	2.9±0.0	12.5±0.1	7.0±0.0	4.1±0.0

Table VIII.5A. *Content of organic acids (µmol L^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *musts at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot A.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
C	**TV**	11.8±0.1	<LOD	779.7±3.9	2,122.8±15.4	14,310.2±14.5	12,655.1±116.3	4,956.4±37.4
	TF	14.1±0.0	<LOD	908.0±25.6	3,105.8±10.3	4,234.0±18.0	19,447.4±11.2	5,048.7±39.9
BABA	**TV**	5.9±0.0	<LOD	909.2±2.2	2,399.1±34.9	9,867.1±112.7	14,966.1±126.8	2,088.9±25.6
	TF	14.8±0.0	<LOD	994.7±23.5	2,044.2±16.9	9,631.9±3.7	15,364.2±40.3	2,540.9±2.9
GT	**TV**	55.4±0.2	520.2±8.5	890.5±30.1	1,813.2±34.1	5,175.1±58.6	13,512.1±63.7	7,239.0±0.8
	TF	21.0±0.0	<LOD	1,626.2±15.7	2,787.7±3.8	5,428.0±3.6	14,790.7±31.1	7,596.2±137.5
BF	**TV**	10.0±0.0	<LOD	1,563.9±7.5	1,837.2±7.1	7,138.9±17.4	12,523.0±35.1	8,472.1±23.4
	TF	12.2±0.0	<LOD	1,376.5±14.5	2,803.4±14.0	5,888.9±23.9	13,755.4±7	4,603.8±24.0
ST	**TV**	8.1±0.0	<LOD	1,052.7±1.6	2,507.5±7.9	9,796.3±13.6	16,120.8±4.4	3,450.6±39.1
	TF	21.2±0.2	<LOD	1,764.5±9.3	2,654.4±19.2	6,779.4±26.7	13,889.2±75.4	4,194.8±5.2

Table VIII.5B. *Content of organic acids (µmol L^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *ra musts at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot B.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
C	**TV**	55.7±0.0	<LOD	1,027.0±4.3	2,439.3±10.2	1,3757.9±17.8	1,5530.5±36.2	4,252.4±18.7
	TF	16.6±0.0	<LOD	1,174.3±15.9	2,342.0±10.7	11,950.8±17.0	1,5082.3±34.1	6,403.8±8.8
BABA	**TV**	14.2±0.0	<LOD	1,100.8±5.7	2,671.0±29.8	13,108±154.7	1,3738.9±73.9	4,468.3±13.5
	TF	28.4±0.1	<LOD	1,353.2±18.0	2,693.5±3.5	11,620.3±8.8	1,3224.0±172.7	4,678.2±28.6
GT	**TV**	69.5±0.3	<LOD	1,170.5±4.0	1,566.4±17.2	17,845.2±11.6	1,4218.0±60.5	3,407.5±7.1
	TF	38.3±0.0	<LOD	1,266.6±14.2	2,888.4±18.4	6,177.9±110.0	1,5143.1±285.3	5,799.5±28.9
BF	**TV**	83.5±0.3	<LOD	1,157.4±21.6	1,715.5±23.5	8,215.5±16.3	1,4783.5±9.9	2,659.6±31.1
	TF	48.0±0.0	<LOD	1,141.3±12.7	1,697.3±18.9	5,722.9±33.9	1,4474.6±23.0	2,742.2±11.1
ST	**TV**	12.3±0.0	<LOD	1,014.3±12.2	2,244.6±51.9	5,743.6±52.8	8,750.1±64.1	3,415.5±41.2
	TF	19.5±0.1	<LOD	1,213.4±8.6	2,308.0±17.0	3,774.9±13.8	14,278.8±125.4	5,909.1±60.4

Table VIII.6A. *Content of organic acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *grapes at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot A.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
C	**TV**	0.0±0.0	<LOD	1.0±0.1	4.7±0.1	11.5±0.1	27.2±0.1	5.6±0.1
	TF	0.0±0.0	<LOD	1.3±0.1	5.0±0.1	8.5±0.2	28.9±0.3	12.2±0.2
BABA	**TV**	0.0±0.0	<LOD	0.8±0.1	3.5±0.1	8.1±0.1	44.3±0.1	2.6±0.1
	TF	0.0±0.0	<LOD	0.7±0.1	3.4±0.1	7.2±0.1	30.5±0.1	3.8±0.1
GT	**TV**	0.0±0.0	<LOD	1.2±0.1	4.3±0.1	7.7±0.1	37±0.1	11.2±0.2
	TF	0.0±0.0	<LOD	1.2±0.1	5.6±0.1	7.1±0.1	31.7±0.1	17.3±0.1
BF	**TV**	0.0±0.0	<LOD	2.7±0.1	4.7±0.1	8.9±0.1	41.2±0.2	5.8±0.1
	TF	0.0±0.0	<LOD	1.5±0.1	4.2±0.1	9.7±0.1	28.0±0.4	9.1±0.1
ST	**TV**	0.0±0.0	<LOD	1.1±0.1	3.7±0.1	10.6±0.1	39.2±0.1	4.5±0.1
	TF	0.0±0.0	<LOD	1.2±0.1	4.5±0.1	10.8±0.1	44.3±0.1	6.1±0.1

***Table VIII.6B**. Content of organic acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *grapes at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot B.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
C	**TV**	0.0±0.0	<LOD	0.9±0.1	3.9±0.1	13.2±0.1	32.1±0.1	2.3±0.1
	TF	0.0±0.0	<LOD	0.9±0.1	4.0±0.1	9.4±0.1	26.2±0.1	4.9±0.1
BABA	**TV**	0.0±0.0	<LOD	1.3±0.1	5.4±0.1	13.8±0.2	48.8±0.2	6.6±0.1
	TF	0.0±0.0	<LOD	1.7±0.1	10.9±0.1	14.6±0.1	44.9±0.1	8.3±0.1
GT	**TV**	0.0±0.0	<LOD	0.9±0.1	5.5±0.1	12.4±0.1	36±0.1	3.7±0.1
	TF	0.0±0.0	<LOD	0.9±0.1	3.5±0.1	8.6±0.1	34.3±0.1	6.8±0.1
BF	**TV**	0.0±0.0	1.7±0.1	1.7±0.1	5.1±0.1	11.7±0.1	42.6±0.2	4.6±0.1
	TF	0.0±0.0	<LOD	1.3±0.1	4.2±0.1	16.5±0.1	17.3±0.2	6.4±0.1
ST	**TV**	0.0±0.0	1.2±0.1	1.5±0.1	4.2±0.1	12.3±0.1	34.3±0.1	2.9±0.1
	TF	0.0±0.0	6.5±0.1	1.9±0.1	4.6±0.1	11.4±0.1	33.4±0.1	6.4±0.1

***Table VIII.7A**. Content of amino acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *leaves after first and 24 hours before first application (T0A and T24B), after and 24 hours before the second foliar application (T0B and T24B) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot A.*

		BABA	GABA	Ala	Arg	Asn	Glu	Asp
C	T0A	0.1±0.0	0.9±0.0	1.0±0.0	0.1±0.0	2.0±0.0	0.6±0.0	0.6±0.0
	T24A	0.1±0.0	8.0±0.0	0.6±0.0	0.1±0.0	1.4±0.0	0.5±0.0	0.4±0.0
	T0B	0.1±0.0	1.3±0.0	1.0±0.0	0.1±0.0	5.2±0.1	1.0±0.0	0.6±0.0
	T24B	<LOD	1.3±0.0	1.2±0.0	<LOD	5±0.2	0.6±0.0	0.4±0.0
	TF	0.1±0.0	0.6±0.0	0.6±0.0	<LOD	8.0±0.0	1.2±0.0	0.6±0.0
BABA	T0A	0.1±0.0	0.6±0.0	0.9±0.0	0.2±0.0	3.4±0.0	0.7±0.0	0.7±0.0
	T24A	0.1±0.0	8.0±0.0	8.0±0.0	0.2±0.0	1.7±0.0	0.4±0.0	8.0±0.0
	T0B	0.1±0.0	1.0±0.0	1.0±0.0	0.2±0.0	8.5±0.2	8.0±0.0	0.6±0.0
	T24B	0.1±0.0	8.0±0.0	1.0±0.0	0.1±0.0	4.5±0.0	8.0±0.0	0.2±0.0
	TF	<LOD	1.4±0.0	1.2±0.0	0.1±0.0	7.8±0.0	1.5±0.0	0.6±0.0
GT	T0A	0.1±0.0	8.0±0.0	0.9±0.0	0.2±0.0	2.2±0.0	0.6±0.0	0.7±0.0
	T24A	0.1±0.0	1.0±0.0	1.2±0.0	0.2±0.0	4.1±0.0	1.0±0.0	0.9±0.0
	T0B	0.1±0.0	1.6±0.0	1.2±0.0	0.2±0.0	8.2±0.0	8.0±0.0	0.6±0.0
	T24B	<LOD	8.0±0.0	1.0±0.0	<LOD	3.6±0.1	0.5±0.0	0.2±0.0
	TF	0.0±0.0	0.3±0.0	0.2±0.0	<LOD	0.7±0.0	0.3±0.0	0.1±0.0
BF	T0A	0.1±0.0	2.4±0.0	1.5±0.0	0.1±0.0	2.0±0.0	0.6±0.0	0.5±0.0
	T24A	0.1±0.0	1.0±0.0	8.0±0.0	0.2±0.0	2.3±0.0	0.5±0.0	0.7±0.0
	T0B	<LOD	1.2±0.0	1.0±0.0	<LOD	4±0.1	0.6±0.0	0.3±0.0
	T24B	<LOD	1.0±0.0	1.3±0.0	<LOD	4.4±0.2	0.6±0.0	0.3±0.0
	TF	0.0±0.0	0.6±0.0	0.4±0.0	<LOD	0.5±0.0	1.2±0.0	0.4±0.0
ST	T0A	0.1±0.0	0.5±0.0	0.6±0.0	0.1±0.0	1.6±0.0	0.6±0.0	0.4±0.0
	T24A	0.1±0.0	0.9±0.0	1.0±0.0	0.2±0.0	2.3±0.0	8.0±0.0	8.0±0.0
	T0B	0.1±0.0	8.0±0.0	0.7±0.0	0.1±0.0	3.0±0.1	0.4±0.0	0.3±0.0
	T24B	0.0±0.0	1.3±0.0	1.8±0.1	<LOD	4.5±0.1	8.0±0.0	0.4±0.0
	TF	0.1±0.0	1.0±0.0	0.7±0.0	<LOD	0.6±0.0	1.0±0.0	0.4±0.0

Table VIII.7B. *Content of amino acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *leaves after first and 24 hours before first application (T0A and T24B), after and 24 hours before the second foliar application (T0B and T24B) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot B.*

		BABA	GABA	Ala	Arg	Asn	Glu	Asp
C	T0A	<LOD	0.7±0.0	0.7±0.0	0.1±0.0	1.6±0.0	1.5±0.0	0.4±0.0
	T24A	0.1±0.0	0.4±0.0	0.6±0.0	<LOD	1.2±0.0	0.5±0.0	0.4±0.0
	T0B	<LOD	<LOD	0.5±0.0	<LOD	0.9±0.0	0.5±0.0	0.4±0.0
	T24B	<LOD	<LOD	0.2±0.0	<LOD	0.1±0.0	0.1±0.0	0.1±0.0
	TF	<LOD	<LOD	0.2±0.0	<LOD	0.1±0.0	0.2±0.0	0.0±0.0
BABA	T0A	<LOD	1.2±0.0	1.0±0.0	<LOD	1.0±0.0	1.0±0.0	0.4±0.0
	T24A	<LOD	0.2±0.0	0.5±0.0	0.0±0.0	2.2±0.0	0.5±0.0	0.6±0.0
	T0B	<LOD	0.0±0.0	8.0±0.0	<LOD	<LOD	0.4±0.0	0.4±0.0
	T24B	<LOD	<LOD	0.2±0.0	<LOD	0.1±0.0	0.2±0.0	0.0±0.0
	TF	<LOD	<LOD	0.3±0.0	<LOD	0.0±0.0	0.4±0.0	0.0±0.0
GT	T0A	<LOD	8.0±0.0	0.7±0.0	<LOD	8.0±0.0	0.9±0.0	0.4±0.0
	T24A	0.1±0.0	<LOD	0.6±0.0	<LOD	0.9±0.0	0.5±0.0	0.3±0.0
	T0B	<LOD	<LOD	0.4±0.0	<LOD	1.9±0.0	0.3±0.0	0.2±0.0
	T24B	<LOD	<LOD	0.2±0.0	<LOD	0.3±0.0	0.2±0.0	0.0±0.0
	TF	<LOD	<LOD	0.3±0.0	<LOD	0.2±0.0	0.2±0.0	0.1±0.0
BF	T0A	0.0±0.0	8.0±0.0	0.5±0.0	<LOD	1.0±0.0	1.0±0.0	0.3±0.0
	T24A	0.0±0.0	<LOD	0.7±0.0	<LOD	0.9±0.0	0.5±0.0	0.5±0.0
	T0B	<LOD	<LOD	0.4±0.0	<LOD	1.9±0.0	0.3±0.0	0.2±0.0
	T24B	<LOD	<LOD	0.1±0.0	<LOD	0.3±0.0	0.2±0.0	<LOD
	TF	<LOD	<LOD	0.2±0.0	<LOD	0.0±0.0	0.2±0.0	<LOD
ST	T0A	0.0±0.0	0.4±0.0	0.6±0.0	<LOD	0.3±0.0	8.0±0.0	0.2±0.0
	T24A	0.0±0.0	<LOD	0.5±0.0	<LOD	8.0±0.0	0.3±0.0	0.5±0.0
	T0B	<LOD	<LOD	0.7±0.0	<LOD	2.2±0.0	0.6±0.0	0.4±0.0
	T24B	<LOD	<LOD	0.1±0.0	<LOD	0.4±0.0	0.2±0.0	0.0±0.0
	TF	<LOD	<LOD	0.1±0.0	<LOD	0.0±0.0	0.1±0.0	0.1±0.0

Table VIII.8A. *Content of amino acids (µmol L^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *musts at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot A.*

		BABA	GABA	Ala	Arg	Asn	Glu	Asp
C	**TV**	2,210.1±52.5	525.1±5.4	6,601.5±113.3	2,186.9±13.5	<LOD	152.4±0.8	97.8±1.6
	TF	2,072.0±15.1	522.0±1.3	6,228.3±13.8	1,465.8±5.7	<LOD	383.7±0.1	147.7±1.3
BABA	**TV**	1,643.6±27.6	451.5±4.8	5,114.0±82.8	1,265.1±14.5	<LOD	147.6±1.6	128.5±0.7
	TF	1,286.5±13.1	339.8±5.0	3,389.9±16.2	679.6±4.8	<LOD	156.8±1.4	86.3±0.6
GT	**TV**	2,127.4±1.4	561.5±17.8	7,226.0±82.7	2,568.4±3.0	<LOD	237.8±0.5	193.4±0.7
	TF	2,291.8±46.3	1,201.2±26.5	12,842.8±275.5	3,197.0±59.7	<LOD	418.9±5.8	158.6±3.7
BF	**TV**	1,997.3±3.7	576.3±15.4	7,006.3±123.9	2,842±34.3	<LOD	178.5±0.1	96.0±0.4
	TF	3,018.3±41.5	551.8±3.4	5,041.5±35.4	1,222.0±10.8	<LOD	351.8±2.8	125.1±1.0
ST	**TV**	1,461.1±18.7	403.9±4.0	4,118.0±25.0	1,435.4±12.9	51±0.5	154.2±1.5	128.3±0.2
	TF	1,438.8±7.8	357.1±5.7	4,454.2±12.9	647.1±2.9	59.1±0.4	117.0±0.5	59.5±0.2

Table VIII.8B. *Content of amino acids (µmol L^{-1}) [Average ± Standard error (SE)]of* Vitis vinifera *musts at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot B.*

		BABA	GABA	Ala	Arg	Asn	Glu	Asp
C	**TV**	2,313.2±15.4	322.3±5.5	8176.6±47.4	1,891.2±18.7	<LOD	136.4±1.8	94.3±1.0
	TF	1459.8±18.7	372.9±4.3	4,346.2±12.2	723.0±0.1	<LOD	230.8±0.2	173.7±2.0
BABA	**TV**	2,672.8±54.0	412.1±3.7	6,809.4±61.9	1,322.3±17.4	<LOD	241.2±2.6	154.8±2.2
	TF	3,087.7±9.3	513.8±2.2	6,426.8±24.3	1,509.3±2.6	<LOD	375.8±2.5	108.5±0.4
GT	**TV**	2,534.9±11.2	454.8±1.6	7,101.6±111.6	2,020.7±34.3	<LOD	278.8±0.3	225.1±1.0
	TF	2,226.2±20.6	448.2±9.4	5,060.1±21.4	965.3±4.7	<LOD	283.1±5.4	100.9±1.3
BF	**TV**	2,450.3±8.6	475.7±3.0	6,708.4±1.7	1,578.1±5.3	<LOD	184.7±2.1	135.1±2.5
	TF	2,609.9±13.1	459.4±4.3	5,862.8±43.6	1,176.6±12.5	<LOD	321.6±2.8	135.1±1.3
ST	**TV**	2,187.4±13.9	463.7±7.7	7,466.6±95.7	2,167.9±39.4	<LOD	199.7±2.9	149.3±1.2
	TF	1,422.9±8.5	367.3±7.3	3,587.5±3.1	438.9±3.0	<LOD	141.9±0.0	78.3±0.3

Table VIII.9A. *Content of amino acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *grapes at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot A.*

		GABA	Ala	Arg	Gln	Asn	Glu	Asp
C	**TV**	0.5±0.1	<LOD	<LOD	0.2±0.1	<LOD	0.6±0.1	0.4±0.1
	TF	0.5±0.1	<LOD	<LOD	0.3±0.1	<LOD	0.4±0.1	0.7±0.1
BABA	**TV**	0.3±0.1	<LOD	<LOD	0.1±0.1	<LOD	0.3±0.1	0.6±0.1
	TF	0.3±0.1	<LOD	<LOD	0.2±0.1	<LOD	0.4±0.1	0.8±0.1
GT	**TV**	0.3±0.1	<LOD	<LOD	0.2±0.1	<LOD	0.5±0.1	0.5±0.1
	TF	0.3±0.1	<LOD	<LOD	0.1±0.1	<LOD	0.2±0.1	0.7±0.1
BF	**TV**	0.4±0.1	<LOD	<LOD	0.1±0.1	<LOD	0.4±0.1	0.9±0.1
	TF	0.3±0.1	<LOD	<LOD	0.1±0.1	<LOD	0.2±0.1	0.7±0.1
ST	**TV**	0.4±0.1	<LOD	<LOD	0.1±0.1	<LOD	0.6±0.1	0.6±0.1
	TF	0.4±0.1	<LOD	<LOD	0.2±0.1	<LOD	0.5±0.1	0.9±0.1

Table VIII.9B. *Content of amino acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *grapes at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2018 in plot B.*

		GABA	Ala	Arg	Gln	Asn	Glu	Asp
C	**TV**	0.8±0.1	0.1±0.1	1.3±0.1	0.3±0.1	<LOD	<LOD	<LOD
	TF	0.3±0.1	<LOD	<LOD	0.1±0.1	<LOD	<LOD	<LOD
BABA	**TV**	1.9±0.1	0.3±0.1	2.5±0.1	0.7±0.1	<LOD	0.2±0.1	1.0±0.1
	TF	1.1±0.1	<LOD	1.5±0.1	0.3±0.1	<LOD	<LOD	<LOD
GT	**TV**	0.3±0.1	<LOD	<LOD	0.1±0.1	<LOD	<LOD	0.8±0.1
	TF	1.5±0.1	0.3±0.1	3±0.1	0.7±0.1	<LOD	<LOD	<LOD
BF	**TV**	0.4±0.1	<LOD	<LOD	0.2±0.1	<LOD	<LOD	1.0±0.1
	TF	1.4±0.1	0.2±0.1	2.2±0.1	0.9±0.1	<LOD	<LOD	<LOD
ST	**TV**	0.8±0.1	0.2±0.1	1.5±0.1	0.3±0.1	<LOD	<LOD	<LOD
	TF	1.1±0.1	0.2±0.1	1.8±0.1	0.4±0.1	<LOD	<LOD	<LOD

Table VIII.10. *Content of carbohydrates (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *leaves after first and 24 hours before first application (T0A and T24B), after and 24 hours before the second foliar application (T0B and T24B) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot A and B.*

		Fruc		Gluc		Sac		Malt	
		A	**B**	**A**	**B**	**A**	**B**	**A**	**B**
C	**T0A**	107.3±3.9	108.5±2.7	129.7±3.7	191.4±1.7	5.5±0.2	<LOD	74.8±2.0	73.5±1.0
	T24A	107.4±1.3	131.4±5.1	195.3±1.8	171.7±0.3	<LOD	<LOD	120.6±1.9	63.5±0.7
	T0B	90.5±0.8	60.4±1.8	96.9±1.7	98.9±3.2	<LOD	<LOD	115.4±2.2	66.4±1.8
	T24B	111.4±2.4	65.9±0.7	133.9±1.9	99.1±2.6	<LOD	<LOD	82.9±1.0	51.8±1.2
	TF	116.9±4.0	89.1±3.0	115.5±2.7	96.5±2.4	24.0±0.7	16.9±0.4	59.9±1.3	83.4±2.0
BABA	**T0A**	91.5±2.9	120.3±1.0	112.4±3.3	159.8±4.5	<LOD	<LOD	81.2±2.0	54.1±0.5
	T24A	100.7±2.0	132.9±4.9	150.6±5.2	197.2±6.9	<LOD	<LOD	65.6±1.9	93.9±0.3
	T0B	95.7±2.8	79.6±1.4	164.1±3.9	141.0±4.6	<LOD	<LOD	117.5±2.5	82.9±1.0
	T24B	103.4±3.4	91.9±3.6	165.9±5.2	157.8±4.6	<LOD	<LOD	110.9±3.4	156.2±5.4
	TF	109.3±3.6	77.2±0.9	99.1±2.9	99.0±1.2	15.0±0.5	28.9±0.7	52.3±0.6	58.7±1.9
GT	**T0A**	84.8±0.1	126.6±3.8	102.6±0.3	203.7±5.4	<LOD	<LOD	31.9±1.2	100.7±3.3
	T24A	109.1±2.9	113.1±1.7	157.5±5.6	184.8±0.2	<LOD	<LOD	102.9±1.1	99.2±2.3
	T0B	122.4±4.6	50.5±1.4	156.8±3.8	74.3±1.6	<LOD	<LOD	155.8±5.0	31.5±1.0
	T24B	63.8±0.4	90.3±2.9	116.4±3.1	166.7±4.9	<LOD	<LOD	98.0±2.5	133.5±3.2
	TF	124.9±3.4	75.9±2.0	142.1±0.9	104.8±2.2	22.5±0.4	6.1±0.2	54.6±2.2	49.0±1.3
BF	**T0A**	79.3±1.7	104.8±3.7	95.9±1.9	179.6±6.2	<LOD	<LOD	<LOD	63.4±0.3
	T24A	108.7±3.7	100.4±2.1	154.6±1.9	183.1±1.6	<LOD	<LOD	55.8±0.3	96.2±2.0
	T0B	112.0±2.4	67.3±2.2	178.6±5.8	128.4±3.5	<LOD	<LOD	82.3±2.3	112.0±3.9
	T24B	81.7±2.8	103.8±0.5	162.4±4.1	159.1±5.8	<LOD	9.2±0.1	86.1±0.8	115.1±3.6
	TF	36.9±0.1	60.4±2.4	49.9±1.5	46.5±1.0	6.8±0.2	2.9±0.2	42.9±0.7	20.2±0.5
ST	**T0A**	110.1±2.3	115.1±1.3	116.1±1.9	159.3±0.6	<LOD	<LOD	33.3±1.1	58.0±1.5
	T24A	73.1±2.3	133.6±0.9	103.8±3.8	188.0±3.2	<LOD	<LOD	40.2±1.5	101.7±3.4
	T0B	92.8±2.3	77.1±2.2	147.9±3.9	103.6±3.3	<LOD	3.8±0.1	114.2±0.9	66.6±2.2
	T24B	115.3±2.9	56.8±1.6	185.6±5.5	117.0±3.1	<LOD	<LOD	82.9±0.6	64.7±1.2
	TF	93.5±2.2	86.9±0.8	105.5±1.7	109.5±3.6	22.6±0.3	16.4±0.3	52.7±0.5	84.1±1.9

Table VIII.11. *Content of carbohydrates (µmol L^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *musts at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot A and B.*

		Fruc		Gluc	
		A	**B**	**A**	**B**
C	**TV**	603,042.8±1,746.8	696,811.9±2,315.2	739,324.4±16,834.5	869,879.2±22,155.5
	TF	610,423.7±4,374.8	639,490.0±2,518.2	671,734.7±1,402.6	784,579.4±1,982.6
BABA	**TV**	610,092.8±2,242.4	581,467.0±6,254.1	764,401.3±13,528.3	630,017.5±5,899.7
	TF	624,087.2±5,944.9	603,857.6±8,490.5	710,873.0±10,303.0	659,499.4±10,945.9
GT	**TV**	567,385.7±8,313.8	645,061.3±3,599.9	752,323.1±8,323.3	775,991.9±14,578.5
	TF	603,373.7±7,014.7	555,417.9±59.7	7,133,443.6±109,807.4	707,395.8±19,403.5
BF	**TV**	570,103.1±2,616.1	647,224.5±12,330.3	677,610.8±4,398.7	706,067.1±6,517.4
	TF	144,799.1±2,767.1	606,994.9±6,801.0	216,662.3±2,789.4	697,530.4±12,309.0
ST	**TV**	598,311.2±3,590.4	565,516.2±4,579.0	723,387.8±18,399.7	691,366.8±6,843.4
	TF	672,313.9±6,928.7	582,010.9±7,568.7	784,571.4±13,709.8	663,818.6±1,840.3

Table VIII.12. *Content of carbohydrates (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *grapes at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot A and B.*

		Fruc		Gluc	
		A	**B**	**A**	**B**
C	**TV**	407.2±0.4	282.4±1.4	458.9±10.5	308.2±5.3
	TF	342.7±3.0	298.0±4.3	386.2±8.3	328.5±0.6
BABA	**TV**	208.6±3.7	282.8±3.4	274.7±1.2	303.9±5.8
	TF	272.4±1.6	334.5±3.4	289.7±1.1	420.5±2.3
GT	**TV**	<LOD	218.5±2.3	<LOD	269.4±1.3
	TF	445.1±3.8	343.3±0.9	472.4±5.2	377.4±7.5
BF	**TV**	<LOD	302.3±3.1	<LOD	364.6±3.5
	TF	486.5±7.9	316.1±0.8	548.3±1.8	352.4±6.3
ST	**TV**	407.2±0.4	282.4±1.4	458.9±10.5	308.2±5.3
	TF	342.7±3.0	298.0±4.3	386.2±8.3	328.5±0.6

Table VIII.13A. *Content of organic acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *leaves after first and 24 hours before first application (T0A and T24B), after and 24 hours before the second foliar application (T0B and T24B) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot A.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
C	T0A	7.2±0.1	0.6±0.0	<LOD	33.5±0.9	0.3±0.0	37.3±0.7	7.2±0.1
	T24A	6.9±0.4	8.0±0.0	4.5±0.2	18.0±0.5	0.3±0.0	28.7±0.1	6.9±0.4
	T0B	4.1±0.0	1.5±0.0	8.0±0.1	51.5±1.7	0.1±0.0	18.9±1.4	4.1±0.0
	T24B	2.2±0.0	1.5±0.1	3.4±0.0	6.5±0.2	0.1±0.0	19.4±0.1	2.2±0.0
	TF	5.7±0.0	1.9±0.0	0.4±0.0	58.4±0.1	0.3±0.0	29.9±0.0	5.7±0.0
BABA	T0A	13.2±0.0	4.2±0.0	0.3±0.0	47±0.2	0.4±0.0	34.0±0.0	13.2±0.0
	T24A	7.7±0.3	2.6±0.2	11.3±0.5	4.1±0.2	0.3±0.0	19.5±0.2	7.7±0.3
	T0B	7.2±0.3	2.1±0.0	1.0±0.0	61.7±1	0.3±0.0	29.7±0.1	7.2±0.3
	T24B	7.9±0.4	2.2±0.0	4.9±0.1	3.9±0.2	0.3±0.0	27.2±0.2	7.9±0.4
	TF	4.3±0.1	1.5±0.0	0.6±0.0	39.3±0.5	0.3±0.0	25.5±1.6	4.3±0.1
GT	T0A	9.7±0.3	2.0±0.0	0.5±0.0	31.6±1.1	0.3±0.0	29.5±0.4	9.7±0.3
	T24A	21.2±0.2	1.0±0.0	2.9±0.0	1.9±0.0	0.6±0.0	29.4±0.3	21.2±0.2
	T0B	6.4±0.2	1.5±0.0	1.4±0.0	73.0±3.9	0.3±0.0	29.5±1.7	6.4±0.2
	T24B	6.7±0.3	4.1±0.3	8.8±0.1	7.1±0.3	0.3±0.0	21.3±0.1	6.7±0.3
	TF	4.4±0.0	8.0±0.0	0.4±0.0	44.7±1	0.2±0.0	22.3±0.3	4.4±0.0
BF	T0A	4.6±0.1	0.9±0.0	0.3±0.0	35.4±0.0	0.3±0.0	24.9±0.6	4.6±0.1
	T24A	3.9±0.2	2.0±0.0	6.5±0.1	5.2±0.2	0.5±0.0	23.9±1.2	3.9±0.2
	T0B	5.3±0.2	2.1±0.1	8.0±0.0	43.4±1.8	0.2±0.0	26.2±1.4	5.3±0.2
	T24B	5.7±0.3	2.6±0.0	6.9±0.2	5.4±0.3	0.2±0.0	19.6±0.4	5.7±0.3
	TF	2.7±0.0	8.0±0.0	0.3±0.0	22.4±0.2	0.2±0.0	17.4±0.6	2.7±0.0
ST	T0A	4.9±0.3	1.2±0.1	<LOD	31.3±2.6	0.2±0.0	23.4±0.6	4.9±0.3
	T24A	9.7±0.3	0.3±0.0	2.1±0.1	7.3±0.2	0.1±0.0	18.4±0.6	9.7±0.3
	T0B	6.8±0.1	3.3±0.1	0.9±0.0	72.2±0.2	0.2±0.0	30.7±0.5	6.8±0.1
	T24B	7.8±0.0	1.3±0.1	3.1±0.0	4.5±0.0	0.2±0.0	29.5±0.8	7.8±0.0
	TF	3.6±0.2	1.9±0.0	0.6±0.0	43.5±0.2	0.2±0.0	19.6±0.9	3.6±0.2

Table VIII.13B. *Content of organic acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *leaves after first and 24 hours before first application (T0A and T24B), after and 24 hours before the second foliar application (T0B and T24B) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot B.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
	T0A	0.0±0.0	1.6±0.0	2.0±0.0	6.4±0.3	0.2±0.0	29.3±0.8	4.6±0.1
	T24A	0.0±0.0	1.8±0.0	0.7±0.0	16.6±0.0	0.2±0.0	15.4±0.4	4.2±0.0
C	**T0B**	0.0±0.0	1.0±0.1	4.7±0.0	9.5±0.2	0.3±0.0	32.0±0.5	6.4±0.2
	T24B	0.0±0.0	3.1±0.1	1.9±0.0	8.6±0.5	0.3±0.0	15.8±0.0	4.0±0.2
	TF	0.0±0.0	1.9±0.0	2.8±0.1	2.8±0.0	0.6±0.0	14.8±0.0	5.0±0.1
	T0A	0.0±0.0	1.7±0.1	2.3±0.0	6±0.2	0.3±0.0	26.5±0.2	5.1±0.0
	T24A	0.0±0.0	1.8±0.0	0.6±0.0	23.8±0.5	0.3±0.0	19.9±0.0	4.0±0.0
BABA	**T0B**	0.0±0.0	1.7±0.0	3.0±0.0	4.2±0.2	0.4±0.0	24.9±0.7	5.8±0.1
	T24B	0.0±0.0	2.6±0.0	1.5±0.0	6.8±0.2	0.3±0.0	12.9±0.4	2.9±0.0
	TF	0.0±0.0	2.0±0.1	2.1±0.0	2.9±0.1	0.4±0.0	10.7±0.2	4.4±0.1
	T0A	0.0±0.0	0.7±0.0	1.9±0.0	5.8±0.2	0.2±0.0	16.5±0.4	3.9±0.1
	T24A	0.0±0.0	1.7±0.0	1.3±0.0	23.0±0.0	0.3±0.0	19.6±0.1	4.6±0.0
GT	**T0B**	0.0±0.0	1.0±0.0	6.1±0.1	10.6±0.0	0.3±0.0	23.6±0.6	5.1±0.2
	T24B	0.0±0.0	3.1±0.1	0.9±0.0	5.1±0.3	0.2±0.0	13.3±0.3	3.2±0.0
	TF	0.0±0.0	1.0±0.0	3.2±0.0	4.3±0.0	0.2±0.0	10.7±0.0	6.2±0.0
	T0A	0.0±0.0	1.4±0.1	2.0±0.1	5.9±0.3	0.2±0.0	18.3±0.3	4.2±0.2
	T24A	0.0±0.0	2.1±0.1	0.4±0.0	33.0±0.1	0.3±0.0	34.8±0.3	5.4±0.0
BF	**T0B**	0.0±0.0	1.5±0.1	2.9±0.1	5.1±0.1	0.4±0.0	24.1±0.3	7.6±0.1
	T24B	0.0±0.0	1.9±0.0	1.0±0.0	3.9±0.1	0.2±0.0	11.3±0.1	2.3±0.0
	TF	0.0±0.0	2.5±0.0	2.4±0.0	2.5±0.1	0.3±0.0	9.0±0.2	5.1±0.1
	T0A	0.0±0.0	1.7±0.1	1.0±0.0	1.3±0.0	0.3±0.0	12.2±0.3	4.7±0.2
	T24A	0.0±0.0	1.2±0.0	0.4±0.0	20.3±0.2	0.2±0.0	11.9±0.2	2.3±0.0
ST	**T0B**	0.0±0.0	0.3±0.0	1.0±0.0	2.4±0.0	0.1±0.0	10.0±0.0	2.8±0.0
	T24B	0.0±0.0	2.0±0.1	0.9±0.0	4.3±0.2	0.1±0.0	11.9±0.7	2.5±0.1
	TF	0.0±0.0	3.4±0.1	2.3±0.0	4.3±0.0	0.3±0.0	4.2.0±0.0	5.5±0.0

Table VIII.14A. *Content of organic acids (µmol L^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *musts at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot A.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
C	**TV**	15.0±0.2	139.0±8.3	2,430.4±5.2	1,122.9±95.8	13,183.8±5.7	12,474.5±40.4	8,824.2±22.0
	TF	29.7±0.1	192.2±4.9	1,461.2±10.9	152.4±9.8	6,765.4±10.6	8,171.7±7.4	8,363.6±2.1
BABA	**TV**	18.0±0.1	103.7±5.1	1,576.8±5.7	424.7±2.9	13,964.5±42.8	8,179.1±15.2	7,008.3±95.0
	TF	21.3±0.1	165.7±1.3	2,168.4±21.8	106.4±3.8	6,794.1±26.8	11,640.1±7.8	3,728.3±9.6
GT	**TV**	19.8±0.1	<LOD	1,656.7±10.1	<LOD	1,5368.5±1.1	10,819.4±3.1	7,880.0±38.9
	TF	24.5±0.1	156.6±2.1	2,533.5±2.4	2,375.8±62.0	11,060.3±44.0	12,195.9±25.6	14,823.9±127.8
BF	**TV**	21.5±0.2	153.9±5.2	2,039.4±0.1	586.8±29.9	10,286.7±32.2	9,001.8±3.4	7,961.6±265.3
	TF	57.8±0.0	272.6±4.6	1,354.0±4.3	481.6±15.8	10,441.5±3.8	10,650.4±7.5	11,027.0±11.7
ST	**TV**	18.9±0.1	155.9±1.5	2,430.4±5.2	1,241.9±27.0	131,837.5±56.7	12,474.5±40.4	88,242.4±219.8
	TF	13.4±5.4	218.0±8.8	1,003±153.2	<LOD	54,567.3±4362.5	7,717.5±151.4	62,520.1±7,038.5

Table VIII.14B. *Content of organic acids (µmol L^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *musts at veraison (TV) and maturity (TF) in* Vitis vinifera *a untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot B.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
C	**TV**	15.6±0.0	398.2±2.2	3,268.9±2.3	2,291.9±14.8	14,058.4±4.4	12,649.5±0.3	16,389.9±2.9
	TF	13.3±0.1	215.4±3.5	1,003.0±11.2	<LOD	5,456.7±4.2	7,717.5±1.3	6,252.0±0.2
BABA	**TV**	109±0.6	448.7±0.3	2,674.3±4.4	365±16.5	8,535.4±1.1	10,611.3±1.9	7,895.7±12.4
	TF	137.8±0.0	465.9±1.2	2,395.0±5.2	<LOD	5,520.3±15.9	10,895.4±1.3	5,021.0±43.0
GT	**TV**	14.3±0.0	89.9±0.1	824.0±3.7	983.7±32.7	9,098.4±2.7	10,222.1±2.8	7,570.0±5.2
	TF	19.2±0.0	179.9±0.7	1,973.8±0.4	696.5±3.3	8,588.6±11.4	10,044.7±0.3	10,511.7±417.4
BF	**TV**	193.5±0.1	962.1±14.4	3,016.3±11.0	<LOD	16,180.5±20.1	12,105.2±9.3	10,167.2±4.1
	TF	292.8±0.1	270.0±8.4	3,048.4±13.4	<LOD	8,585.9±4.5	9,410.7±5.6	7,833.2±7.7
ST	**TV**	18.9±0.1	200.7±183.7	1,442.3±1,480.1	169.4±159.1	67,470.7±67,837.5	8,184.4±8,158.9	83,599.9±83,671.4
	TF	23.3±0.0	267.6±3.2	2452.1±1.9	<LOD	4,976.0±10.1	13,677.0±9.0	7,933.0±0.8

Table VIII.15A. *Content of organic acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *grapes at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot A.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
C	**TV**	0.0±0.0	1.5±0.1	0.9±0.1	1.0±0.1	22.1±0.1	16.3±0.1	10.3±0.3
	TF	0.0±0.0	3.5±0.2	1.8±0.1	1.3±0.1	19.0±0.1	23.0±0.2	23.3±0.9
BABA	**TV**	0.0±0.0	1.0±0.1	0.6±0.1	1.1±0.1	17.0±0.1	13.7±0.1	14.8±0.2
	TF	0.0±0.0	1.3±0.1	1.1±0.1	0.3±0.1	14.4±0.1	19.6±0.2	12.9±0.1
GT	**TV**	0.0±0.0	1.3±0.1	0.9±0.1	2.0±0.1	18.6±0.1	20.4±0.1	12.7±0.2
	TF	0.1±0.0	1.3±0.1	1.5±0.1	1.1±0.1	14.4±0.2	18.9±0.1	14.2±0.6
BF	**TV**	0.0±0.0	1.1±0.1	0.9±0.1	0.8±0.1	16.2±0.1	13.9±0.1	12.7±0.5
	TF	0.0±0.0	0.9±0.1	1.3±0.1	<LOD	17.7±0.1	16.5±0.1	16.4±0.2
ST	**TV**	0.0±0.0	1.7±0.1	1.4±0.1	1.0±0.1	23.9±0.1	19.4±0.1	11.5±0.3
	TF	0.0±0.0	6.7±0.1	1.7±0.1	0.8±0.1	20.3±0.1	22.9±0.1	20.4±0.7

Table VIII.15B. *Content of organic acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *grapes at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot B.*

		Fum	Succ	Cit	Acet	Mal	Tart	Oxal
C	**TV**	0.0±0.0	1.5±0.1	1.3±0.1	1.1±0.1	20.7±0.3	20.0±0.2	19.5±0.5
	TF	0.0±0.0	0.7±0.1	1.1±0.1	0.3±0.1	15.5±0.1	18.5±0.1	13.0±0.3
BABA	**TV**	0.0±0.0	1.4±0.1	1.2±0.1	1.1±0.1	26.3±0.1	19.8±0.1	15.5±0.7
	TF	0.0±0.0	0.9±0.1	1.2±0.1	<LOD	16.0±0.1	15.9±0.1	14.3±0.6
GT	**TV**	0.0±0.0	0.8±0.1	0.7±0.1	1.4±0.1	13.6±0.1	17.7±0.1	10.9±0.4
	TF	0.0±0.0	1.1±0.1	1.5±0.1	0.2±0.1	19.6±0.1	19.8±0.1	16.7±0.8
BF	**TV**	0.0±0.0	1.6±0.1	1.7±0.1	1.4±0.1	24.8±0.2	20.2±0.1	17.2±0.5
	TF	0.0±0.0	0.9±0.1	1.2±0.1	<LOD	14.1±0.1	18.4±0.1	17.5±0.1
ST	**TV**	0.0±0.0	1.3±0.1	0.9±0.1	2.3±0.1	21.4±0.1	19.6±0.1	11.0±0.4
	TF	0.0±0.0	4.4±0.1	1.1±0.1	0.1±0.1	12.1±0.2	17.7±0.1	13.9±0.8

Table VIII.16A. *Content of amino acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *leaves after first and 24 hours before first application (T0A and T24B), after and 24 hours before the second foliar application (T0B and T24B) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot A.*

		BABA	GABA	Ala	Arg	Asn	Glu	Asp
C	T0A	0.4±0.0	4.0±0.1	1.2±0.0	0.0±0.0	2.2±0.0	0.4±0.0	0.1±0.0
	T24A	0.3±0.0	2.6±0.0	1.4±0.0	0.1±0.0	2.2±0.0	0.5±0.0	0.2±0.0
	T0B	0.3±0.0	3.2±0.0	2.1±0.0	0.1±0.0	1.6±0.0	0.3±0.0	0.1±0.0
	T24B	0.1±0.0	1.4±0.0	0.7±0.0	0.1±0.0	1.7±0.0	0.6±0.0	0.2±0.0
	TF	0.1±0.0	0.5±0.0	0.3±0.0	0.1±0.0	0.7±0.0	0.1±0.0	0.1±0.0
BABA	T0A	0.2±0.0	1.9±0.0	8.0±0.0	0.2±0.0	7.2±0.0	0.6±0.0	0.2±0.0
	T24A	0.2±0.0	2.3±0.1	1.2±0.0	0.2±0.0	5.3±0.1	0.4±0.0	0.2±0.0
	T0B	0.3±0.0	3.3±0.0	2.6±0.0	0.1±0.0	1.8±0.0	0.3±0.0	0.2±0.0
	T24B	0.3±0.0	3.1±0.0	1.4±0.0	0.1±0.0	3.2±0.0	0.6±0.0	0.2±0.0
	TF	0.1±0.0	0.3±0.0	0.6±0.0	0.1±0.0	0.3±0.0	0.3±0.0	0.2±0.0
GT	T0A	0.4±0.0	3.7±0.0	1.9±0.0	0.1±0.0	5.7±0.0	0.7±0.0	0.2±0.0
	T24A	0.1±0.0	1.0±0.0	0.6±0.0	0.0±0.0	2.3±0.0	0.6±0.0	0.3±0.0
	T0B	0.4±0.0	3.4±0.0	2.4±0.0	0.1±0.0	3.3±0.1	8.0±0.0	0.2±0.0
	T24B	0.3±0.0	2.7±0.0	2.5±0.0	0.0±0.0	1.2±0.0	0.2±0.0	0.1±0.0
	TF	0.1±0.0	0.9±0.0	0.9±0.0	0.1±0.0	1.0±0.0	0.4±0.0	0.2±0.0
BF	T0A	0.3±0.0	2.7±0.0	1.5±0.0	0.0±0.0	2.8±0.0	8.0±0.0	0.3±0.0
	T24A	0.2±0.0	1.9±0.0	0.9±0.0	0.2±0.0	4.1±0.1	0.3±0.0	0.2±0.0
	T0B	0.3±0.0	3.5±0.0	1.3±0.0	0.1±0.0	1.0±0.0	0.4±0.0	0.1±0.0
	T24B	0.3±0.0	3.3±0.0	2.1±0.0	0.1±0.0	2.0±0.0	0.9±0.0	0.2±0.0
	TF	0.1±0.0	0.3±0.0	0.5±0.0	0.0±0.0	0.2±0.0	0.2±0.0	0.2±0.0
ST	T0A	0.2±0.0	2.2±0.0	1.0±0.0	0.1±0.0	6.1±0.1	0.6±0.0	0.3±0.0
	T24A	0.1±0.0	8.0±0.0	0.4±0.0	0.0±0.0	1.5±0.0	0.2±0.0	0.2±0.0
	T0B	0.2±0.0	1.8±0.0	1.4±0.0	0.1±0.0	1.6±0.0	0.2±0.0	0.1±0.0
	T24B	0.3±0.0	4.0±0.1	2.0±0.0	0.1±0.0	1.8±0.0	0.6±0.0	0.2±0.0
	TF	0.1±0.0	0.5±0.0	0.5±0.0	0.1±0.0	0.7±0.0	0.3±0.0	0.1±0.0

Table VIII.16B. *Content of amino acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *leaves after first and 24 hours before first application (T0A and T24B), after and 24 hours before the second foliar application (T0B and T24B) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot B.*

		BABA	GABA	Ala	Arg	Asn	Glu	Asp
C	T0A	0.1±0.0	0.9±0.0	0.6±0.0	0.1±0.0	0.6±0.0	0.2±0.0	0.1±0.0
	T24A	0.0±0.0	0.3±0.0	0.4±0.0	0.0±0.0	0.3±0.0	0.2±0.0	0.2±0.0
	T0B	0.0±0.0	0.4±0.0	0.7±0.0	0.1±0.0	0.4±0.0	0.3±0.0	0.3±0.0
	T24B	0.0±0.0	0.7±0.0	0.3±0.0	0.1±0.0	0.3±0.0	0.2±0.0	0.5±0.0
	TF	0.0±0.0	8.0±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.5±0.0
BABA	T0A	0.1±0.0	0.9±0.0	1.2±0.0	0.1±0.0	0.6±0.0	0.5±0.0	0.2±0.0
	T24A	0.0±0.0	0.5±0.0	0.6±0.0	0.1±0.0	0.1±0.0	0.3±0.0	0.2±0.0
	T0B	0.0±0.0	1.0±0.0	1.5±0.0	0.1±0.0	0.7±0.0	0.5±0.0	0.5±0.0
	T24B	0.0±0.0	1.0±0.0	0.3±0.0	0.2±0.0	0.6±0.0	0.3±0.0	0.6±0.0
	TF	0.0±0.0	8.0±0.0	0.4±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.7±0.0
GT	T0A	0.1±0.0	0.4±0.0	0.7±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.0
	T24A	0.0±0.0	0.3±0.0	0.6±0.0	0.0±0.0	0.0±0.0	0.3±0.0	0.3±0.0
	T0B	0.0±0.0	0.5±0.0	1.4±0.0	0.1±0.0	0.4±0.0	0.7±0.0	0.5±0.0
	T24B	0.0±0.0	1.3±0.0	0.5±0.0	0.2±0.0	0.7±0.0	0.3±0.0	8.0±0.0
	TF	0.0±0.0	0.6±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.6±0.0
BF	T0A	0.1±0.0	0.7±0.0	0.6±0.0	0.1±0.0	0.4±0.0	0.3±0.0	0.2±0.0
	T24A	0.0±0.0	0.6±0.0	1.3±0.0	0.1±0.0	0.4±0.0	0.7±0.0	0.3±0.0
	T0B	0.0±0.0	0.3±0.0	1.0±0.0	0.1±0.0	0.3±0.0	0.6±0.0	0.4±0.0
	T24B	0.0±0.0	1.0±0.0	0.4±0.0	0.2±0.0	0.3±0.0	0.3±0.0	8.0±0.0
	TF	0.0±0.0	1.0±0.0	0.3±0.0	0.2±0.0	0.6±0.0	0.3±0.0	0.6±0.0
ST	T0A	0.0±0.0	0.3±0.0	0.3±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.1±0.0
	T24A	0.0±0.0	0.5±0.0	1.0±0.0	0.1±0.0	0.5±0.0	8.0±0.0	0.3±0.0
	T0B	0.0±0.0	0.4±0.0	0.9±0.0	0.0±0.0	0.1±0.0	0.4±0.0	0.4±0.0
	T24B	0.0±0.0	8.0±0.0	0.3±0.0	0.2±0.0	0.4±0.0	0.2±0.0	0.6±0.0
	TF	0.0±0.0	1.0±0.0	0.2±0.0	0.1±0.0	0.4±0.0	0.3±0.0	0.5±0.0

Table VIII.17A. *Content of amino acids (µmol L^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *musts at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot A.*

		BABA	GABA	Ala	Arg	Asn	Glu	Asp
C	**TV**	803.7±8.6	152.4±2.5	1,776.4±18.7	294.2±7.0	3.7±0.0	18.6±0.1	15.4±0.0
	TF	1,680.4±19	814.2±10.3	7,899.2±4.8	3265.5±6.5	41.8±0.2	213.6±0.6	120.3±0.1
BABA	**TV**	2,672.8±54.0	412.1±3.7	6,809.4±61.9	1322.3±17.4	5.0±0.0	241.2±2.6	154.8±2.2
	TF	3,087.7±9.3	513.8±2.2	6,426.8±24.3	1509.3±2.6	9.9±0.0	375.8±2.5	108.5±0.4
GT	**TV**	1,633.1±3.0	845.4±14.2	5,099.7±25.9	1209.6±11.8	20.7±0.1	226.0±5.0	257.4±9.9
	TF	1,481.0±38.4	661.9±15.3	5,908.1±25.3	1088.3±9.8	26.8±0.1	141.2±2	110.7±0.5
BF	**TV**	1,267.4±4.8	689.3±6.7	3,811.4±1.9	790.3±2.8	10.5±0.0	157.8±0.8	165.3±1.1
	TF	1,711.5±15.7	504.4±5.3	5,284.3±14.7	694.1±7.9	22.1±0.1	84.3±1.6	95.1±0.8
ST	**TV**	937.3±0.1	237.1±1.6	2,794.6±4.8	575.3±3.1	3.7±0.0	51.1±0.8	26.7±0.0
	TF	1,995.5±22.2	718.6±7.9	6,240.4±13.3	1440.7±4.6	22.6±0.2	178.0±0.1	125.5±0.0

Table VIII.17B. *Content of amino acids (µmol L^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *ra musts at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot B.*

		BABA	GABA	Ala	Arg	Asn	Glu	Asp
C	**TV**	1,499.1±25.2	1129±34.1	7,941.0±28.5	3877±16.6	51.3±0.2	480.3±1.8	237.6±6.9
	TF	1,353.1±7.2	510.0±7.8	4,600.0±3.2	971.3±0.7	17.7±0.2	132.3±0.3	140.7±0.1
BABA	**TV**	1,444.4±16.0	838.8±4.6	6,501.6±64.1	1559.5±14.7	49.6±0.6	196.0±1.4	213.0±0.0
	TF	1,634.1±1.4	682.6±1.5	6,120.7±7.5	976.7±1.8	2.4±0.0	183.0±2.3	94.9±0.3
GT	**TV**	1,416.4±16.1	586.0±0.9	4,295.6±48.6	1345.1±11.6	15.8±0.0	187.8±1.6	130.5±1.7
	TF	1,518.2±3.5	487.1±7.7	4,208.5±3.4	767.1±12.4	16.0±0.1	129.5±0.2	86.0±0.8
BF	**TV**	1,996.7±31.6	1,733.1±1.9	9,942.2±10.1	2715.6±5.3	106±0.3	392.1±1.1	232.2±1.8
	TF	1,685.8±3.8	597.9±10	5,867.3±9.8	881.9±0.2	26.3±0.0	162.4±0.3	140.9±0.5
ST	**TV**	1,555.7±15.2	690.1±7.1	5,294.5±36.3	1594.0±2.8	15.1±0.0	180.8±3.6	100.4±1.2
	TF	1,630.7±7.8	603.9±9.2	5,943.3±6.4	1284.2±2.3	20.2±0.1	187.5±1.4	121.0±0.1

Table VIII.18A. *Content of amino acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *grapes at veraison (TV) and maturity (TF)* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot A.*

		GABA	Ala	Arg	Gln	Asn	Glu	Asp
C	**TV**	0.8±0.1	0.6±0.1	2.8±0.1	1.1±0.1	0.2±0.1	0.1±0.1	0.6±0.1
	TF	1.6±0.1	1.1±0.1	6.1±0.1	5.0±0.1	0.2±0.1	0.3±0.1	1.0±0.1
BABA	**TV**	1.1±0.1	0.9±0.1	4.6±0.1	1.7±0.1	0.2±0.1	0.2±0.1	0.7±0.1
	TF	1.0±0.1	0.4±0.1	3.9±0.1	0.6±0.1	<LOD	<LOD	0.7±0.1
GT	**TV**	1.6±0.1	1.1±0.1	5.1±0.1	1.7±0.1	0.3±0.1	0.4±0.1	1.6±0.1
	TF	2.1±0.1	0.7±0.1	4.9±0.1	1.1±0.1	0.1±0.1	<LOD	0.9±0.1
BF	**TV**	1.1±0.1	1.0±0.1	6.0±0.1	1.4±0.1	0.2±0.1	0.3±0.1	1.9±0.1
	TF	1.6±0.1	0.7±0.1	6.3±0.1	1.2±0.1	0.1±0.1	<LOD	0.9±0.1
ST	**TV**	0.9±0.1	0.6±0.1	2.9±0.1	0.9±0.1	0.2±0.1	0.1±0.1	0.5±0.1
	TF	1.4±0.1	0.6±0.1	5.8±0.1	1.4±0.1	0.1±0.1	0.2±0.1	1.1±0.1

Tabla VIII.18B. *Content of amino acids (µmol g^{-1}) [Average ± Standard error (SE)] of* Vitis vinifera *grapes at veraison (TV) and maturity (TF) in* Vitis vinifera *untreated (Control) or treated with BABA or three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown in 2019 in plot B.*

		GABA	Ala	Arg	Gln	Asn	Glu	Asp
C	**TV**	1.3±0.1	1.3±0.1	7.0±0.1	4.5±0.1	<LOD	0.6±0.1	1.9±0.1
	TF	1.2±0.1	0.4±0.1	4.7±0.1	0.8±0.1	0.1±0.1	<LOD	0.9±0.1
BABA	**TV**	1.5±0.1	1.2±0.1	6.4±0.1	2.2±0.1	<LOD	0.2±0.1	1.5±0.1
	TF	1.5±0.1	0.5±0.1	5.4±0.1	0.8±0.1	0.3±0.1	<LOD	0.7±0.1
GT	**TV**	1.1±0.1	0.5±0.1	4.3±0.1	1.1±0.1	<LOD	0.1±0.1	1.0±0.1
	TF	1.3±0.1	0.3±0.1	4.7±0.1	1.0±0.1	0.2±0.1	<LOD	0.6±0.1
BF	**TV**	1.7±0.1	1.3±0.1	6.8±0.1	2.6±0.1	<LOD	0.4±0.1	1.1±0.1
	TF	1.3±0.1	0.3±0.1	3.1±0.1	0.9±0.1	<LOD	<LOD	<LOD
ST	**TV**	1.2±0.1	0.6±0.1	4.7±0.1	1.4±0.1	<LOD	0.2±0.1	1.4±0.1
	TF	1.4±0.1	0.5±0.1	4.8±0.2	1.4±0.1	<LOD	0.1±0.1	0.8±0.1

Table VIII.19. *Content of free amino acids and organic acids (mg L^{-1}) in the three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] ± Standard error (SE).*

	GT	BF	ST
Asp	14.7±0.1	104.6±0.1	12.9±0.1
Glu	78.6±0.0	405.1±0.2	100.7±0.1
Asn	0.3±0.0	1.2±0.1	0.2±0.1
Gln	1.5±0.0	0.8±0.1	0.9±0.0
Arg	1.0±0.0	89.6±0.1	0.8±0.1
Ala	16.5±0.1	116.5±0.0	15.5±0.1
GABA	2.1±0.1	14.9±0.0	1.6±0.1
BABA	0.5±0.0	204.1±0.1	0.3±0.0
Oxal	3,201.3±28.4	2,123.7±15.2	4,507.4±5.8
Tart	440.1±0.1	606.1±0.3	1,060.5±23.5
Mal	<LOD	612.2±0.1	97.1±0.1
Acet	42.8±0.2	20.2±0.1	18.3±0.1
Cit	172.2±0.1	993.0±0.0	8.5±0.3
Succ	<LOD	<LOD	<LOD
Fum	5.6±0.1	9.5±0.1	22.3±0.1

ANNEX IX: RESULTS OF *ARABIDOPSIS THALIANA* PHENOTYPING PARAMETERS.

Table IX.1. *Chlorophyll fluorescence parameters [average ± Standard error (SE)] in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field water capacity (FC) or progressive drought (PD).*

		Days after Sowing (DAS)	QY_max	Fv/Fm_Lss	QY_Lss	NPQ_Lss	qN_Lss	qP_Lss	qL_Lss	Rfd_Lss
FC	C	26	0.8±9.0.10-4	0.6±4.2.10-3	0.6±1.7.10-3	0.3±1.8.10-2	0.3±1.1.10-2	0.9±4.1.10-3	1.1±4.6.10-3	2.3±1.8.10-1
		28	0.8±5.0.10-4	0.6±2.3.10-3	0.5±2.6.10-3	0.4±1.4.10-2	0.4±8.6.10-3	0.9±3.3.10-2	1.0±2.1.10-2	1.9±1.5.10-1
		33	0.8±8.0.10-4	0.6±2.3.10-3	0.5±3.2.10-3	0.4±1.2.10-2	0.3±9.4.10-3	0.9±4.2.10-3	1.1±2.1.10-3	1.6±5.4.10-2
		35	0.8±7.0.10-4	0.6±3.6.10-3	0.5±3.9.10-3	0.3±8.5.10-3	0.3±7.1.10-3	0.9±2.0.10-3	1.1±2.7.10-3	1.2±3.1.10-2
		40	0.8±6.0.10-3	0.6±1.0.10-2	0.6±7.5.10-3	0.4±5.8.10-2	0.4±3.6.10-2	0.9±1.2.10-2	1.0±8.5.10-3	1.6±1.4.10-1
		42	0.8±5.1.10-3	0.6±5.7.10-3	0.6±6.2.10-3	0.3±2.1.10-2	0.3±1.6.10-2	0.9±4.1.10-3	1.1±4.2.10-3	1.4±9.2.10-2
	GT	26	0.8±1.5.10-3	0.6±9.2.10-3	0.6±4.1.10-3	0.4±3.0.10-2	0.4±2.0.10-2	0.9±9.9.10-3	1.0±1.2.10-2	2.8±1.7.10-1
		28	0.8±1.0.10-3	0.6±2.0.10-3	0.5±3.4.10-3	0.4±1.1.10-2	0.4±5.9.10-3	0.9±1.4.10-2	1.0±7.9.10-3	2.1±1.3.10-1
		33	0.8±9.0.10-4	0.6±2.0.10-3	0.5±1.8.10-3	0.3±9.9.10-3	0.3±7.9.10-3	0.9±4.3.10-3	1.1±2.3.10-3	1.4±4.9.10-2
		35	0.8±1.1.10-3	0.6±2.9.10-3	0.5±4.7.10-3	0.4±1.2.10-2	0.4±7.8.10-3	0.9±8.1.10-3	1.1±2.8.10-3	1.5±7.2.10-2
		40	0.8±9.0.10-4	0.6±7.2.10-3	0.6±8.7.10-3	0.4±3.1.10-2	0.4±2.0.10-2	0.9±1.0.10-2	1.0±5.3.10-3	1.4±7.7.10-2
		42	0.8±1.0.10-3	0.6±1.1.10-2	0.6±5.9.10-3	0.4±5.2.10-2	0.4±3.3.10-2	0.9±9.0.10-3	1.0±9.2.10-3	1.6±9.7.10-2
	BF	26	0.8±1.6.10-3	0.7±3.4.10-3	0.6±3.2.10-3	0.3±1.3.10-2	0.3±7.2.10-3	0.9±3.9.10-3	1.1±5.8.10-3	2.6±1.5.10-1
		28	0.8±6.0.10-4	0.6±1.1.10-3	0.5±1.2.10-3	0.3±7.8.10-3	0.3±5.4.10-3	0.9±2.3.10-3	1.1±1.7.10-3	1.3±6.4.10-2
		33	0.8±2.1.10-3	0.6±2.2.10-3	0.6±4.1.10-3	0.3±6.6.10-3	0.3±5.1.10-3	0.9±6.0.10-3	1.1±2.9.10-3	1.2±4.3.10-2
		35	0.8±4.1.10-3	0.6±1.8.10-3	0.6±1.6.10-3	0.3±8.2.10-3	0.3±6.0.10-3	0.9±2.8.10-3	1.1±1.9.10-3	1.8±6.3.10-2
		40	0.8±4.2.10-3	0.6±3.5.10-3	0.6±9.9.10-3	0.3±1.9.10-2	0.3±2.6.10-2	0.9±1.6.10-2	1.0±6.7.10-3	1.9±1.3.10-1
		42	0.8±3.2.10-3	0.6±3.7.10-3	0.6±5.1.10-3	0.3±2.4.10-2	0.3±1.7.10-2	0.9±4.6.10-3	1.1±3.1.10-3	1.3±1.0.10-1
	ST	26	0.8±1.1.10-3	0.7±3.6.10-3	0.6±3.3.10-3	0.3±1.5.10-2	0.3±8.6.10-3	0.9±3.8.10-3	1.1±7.3.10-3	2.2±1.3.10-1
		28	0.8±7.0.10-4	0.6±1.4.10-3	0.5±1.2.10-3	0.3±1.2.10-2	0.3±9.5.10-3	0.9±2.2.10-3	1.1±2.5.10-3	1.5±8.9.10-2
		33	0.8±8.0.10-4	0.6±3.7.10-3	0.5±3.3.10-3	0.4±1.2.10-2	0.3±1.8.10-2	0.9±3.9.10-3	1.1±2.9.10-3	1.5±6.0.10-2
		35	0.8±8.0.10-4	0.6±2.8.10-3	0.5±3.2.10-3	0.3±1.0.10-2	0.4±7.4.10-3	0.9±3.8.10-3	1.1±2.2.10-3	1.4±2.8.10-2
		26	0.8±1.1.10-3	0.7±3.6.10-3	0.6±3.3.10-3	0.3±1.5.10-2	0.3±8.6.10-3	0.9±3.8.10-3	1.1±7.3.10-3	2.2±1.3.10-1
		28	0.8±7.0.10-4	0.6±1.4.10-3	0.5±1.2.10-3	0.3±1.2.10-2	0.3±9.5.10-3	0.9±2.2.10-3	1.1±2.5.10-3	1.5±8.9.10-2

Table IX.1 (Cont.). *Chlorophyll fluorescence parameters [average ± Standard error (SE)] in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field water capacity (FC) or progressive drought (PD).*

		Days after Sowing (DAS)	QY_max	Fv/Fm_Lss	QY_Lss	NPQ_Lss	qN_Lss	qP_Lss	qL_Lss	Rfd_Lss
PD	C	26	0.8±1.0.10-3	0.6±5.8.10-3	0.6±3.7.10-3	0.4±2.1.10-2	0.3±1.3.10-2	0.9±5.5.10-3	1.1±5.3.10-3	2.5±1.6.10-1
		28	0.8±9.0.10-4	0.6±2.4.10-3	0.5±2.5.10-3	0.4±1.4.10-2	0.4±9.3.10-3	0.9±5.3.10-3	1.1±2.2.10-3	1.9±1.0.10-1
		33	0.8±9.0.10-4	0.6±4.0.10-3	0.6±3.5.10-3	0.4±2.3.10-2	0.4±1.4.10-2	0.9±3.1.10-3	1.0±2.3.10-3	1.5±6.6.10-2
		35	0.8±1.0.10-3	0.6±4.7.10-3	0.5±3.9.10-3	0.4±1.8.10-2	0.4±1.4.10-2	0.9±3.5.10-3	1.1±3.7.10-3	1.4±6.3.10-2
		40	0.8±1.5.10-3	0.6±4.8.10-3	0.6±7.1.10-3	0.3±1.7.10-2	0.3±1.3.10-2	0.9±7.8.10-3	1.1±4.9.10-3	1.5±6.6.10-2
		42	0.8±2.3.10-3	0.6±4.7.10-3	0.6±1.0.10-2	0.3±2.4.10-2	0.3±1.7.10-2	0.9±1.1.10-2	1.1±4.7.10-3	1.9±1.3.10-1
	GT	26	0.8±1.1.10-3	0.7±2.1.10-3	0.6±2.5.10-3	0.3±1.1.10-2	0.3±5.4.10-3	0.9±3.4.10-3	1.1±2.7.10-3	2.4±1.7.10-1
		28	0.8±7.0.10-4	0.6±1.5.10-3	0.5±1.6.10-3	0.3±9.8.10-3	0.3±5.1.10-3	0.9±3.1.10-3	1.1±3.2.10-3	1.6±8.5.10-2
		33	0.8±7.0.10-4	0.6±3.5.10-3	0.5±1.6.10-3	0.4±2.0.10-2	0.4±1.2.10-2	0.9±4.3.10-3	1.0±2.8.10-3	1.4±3.7.10-2
		35	0.8±8.0.10-4	0.6±2.0.10-3	0.5±3.4.10-3	0.3±1.1.10-2	0.3±7.0.10-3	0.9±6.7.10-3	1.1±2.4.10-3	1.2±2.7.10-2
		40	0.8±5.0.10-4	0.6±5.1.10-3	0.6±2.5.10-3	0.3±2.4.10-2	0.3±2.0.10-2	0.9±9.1.10-3	1.1±6.5.10-3	1.4±4.3.10-2
		42	0.8±2.4.10-3	0.6±3.9.10-3	0.6±5.3.10-3	0.3±1.0.10-2	0.3±6.9.10-3	0.9±1.3.10-2	1.1±8.4.10-3	1.3±4.9.10-2
	BF	26	0.8±6.0.10-4	0.7±2.3.10-3	0.6±2.7.10-3	0.3±9.8.10-3	0.3±5.9.10-3	0.9±7.0.10-3	1.1±9.3.10-3	2.1±1.1.10-1
		28	0.8±5.0.10-4	0.6±2.4.10-3	0.5±1.9.10-3	0.4±1.7.10-2	0.3±9.5.10-3	0.8±1.8.10-2	1.0±2.4.10-2	1.8±1.4.10-1
		33	0.8±9.0.10-4	0.6±2.9.10-3	0.6±2.0.10-3	0.3±1.4.10-2	0.3±9.4.10-3	0.9±5.9.10-3	1.1±3.9.10-3	1.5±4.7.10-2
		35	0.8±1.4.10-3	0.6±2.6.10-3	0.6±2.2.10-3	0.3±8.2.10-3	0.3±6.6.10-3	0.9±4.8.10-3	1.1±3.3.10-3	1.2±3.3.10-2
		40	0.8±1.7.10-3	0.7±5.9.10-3	0.6±5.7.10-3	0.3±1.2.10-2	0.3±1.1.10-2	0.9±6.1.10-3	1.1±5.7.10-3	1.4±4.3.10-2
		42	0.8±3.2.10-3	0.7±6.7.10-3	0.6±7.4.10-3	0.3±1.6.10-2	0.3±1.3.10-2	0.9±1.5.10-2	1.1±7.8.10-3	1.8±8.6.10-2
	ST	26	0.8±3.5.10-3	0.6±4.4.10-3	0.6±1.7.10-3	0.3±1.2.10-2	0.3±8.0.10-3	0.9±6.2.10-3	1.0±4.5.10-3	1.9±9.5.10-2
		28	0.8±7.0.10-4	0.6±2.4.10-3	0.5±2.1.10-3	0.3±1.1.10-2	0.3±7.7.10-3	0.9±5.8.10-3	1.1±9.5.10-3	1.9±1.0.10-1
		33	0.8±4.0.10-4	0.6±2.9.10-3	0.5±2.0.10-3	0.4±1.3.10-2	0.4±7.4.10-3	0.9±3.2.10-3	1.0±2.1.10-3	1.5±5.4.10-2
		35	0.8±6.0.10-4	0.6±3.3.10-3	0.5±2.2.10-3	0.3±1.6.10-2	0.3±1.3.10-2	0.9±3.9.10-3	1.1±3.3.10-3	1.3±4.3.10-2
		40	0.8±1.0.10-3	0.6±3.6.10-3	0.6±4.8.10-3	0.3±1.5.10-2	0.3±1.1.10-2	0.9±6.5.10-3	1.1±3.6.10-3	1.4±6.0.10-2
		42	0.8±1.3.10-3	0.6±3.6.10-3	0.6±4.2.10-3	0.3±1.5.10-2	0.3±1.2.10-2	0.9±5.3.10-3	1.0±4.8.10-3	1.4±7.1.10-2

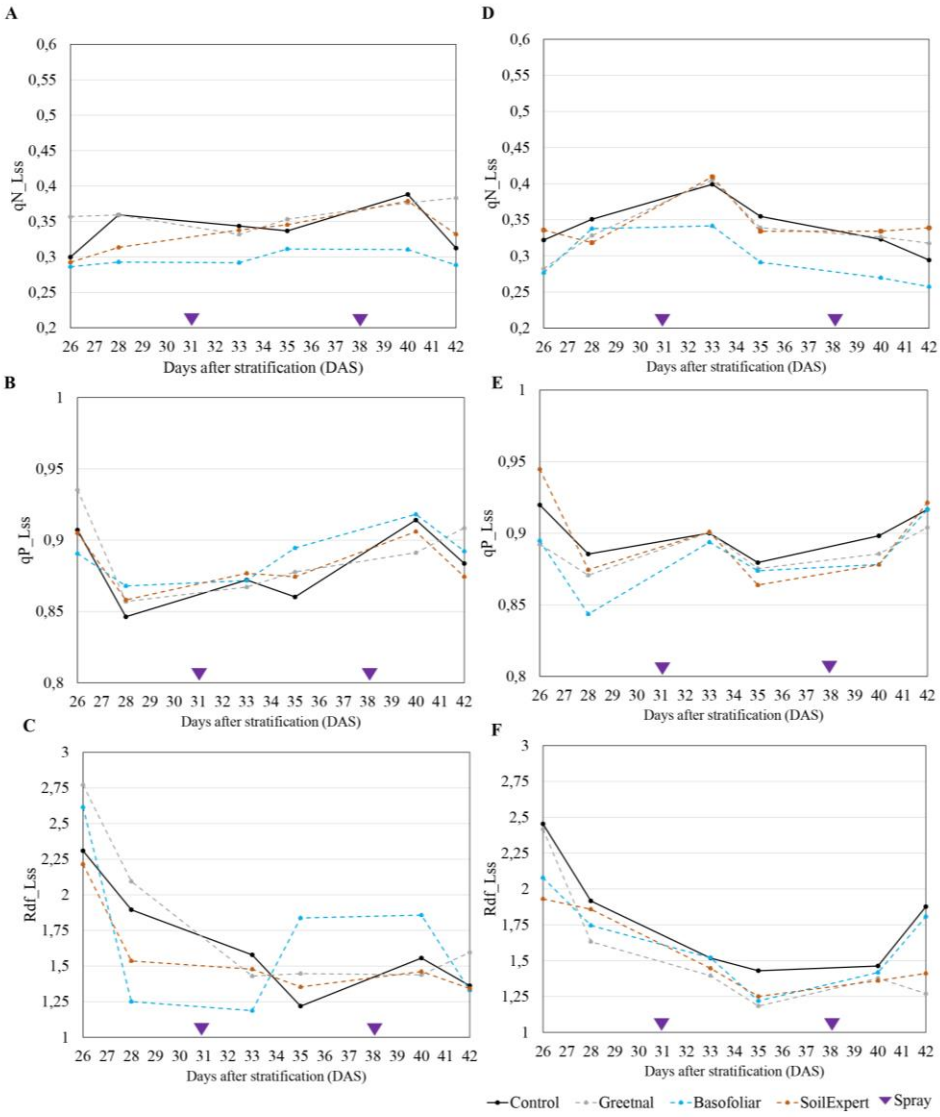

Figure IX.1. *Chlorophyll fluorescence parameters [average ± Standard error (SE)] in Arabidopsis seedlings untreated (Control) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field water capacity (FC) or progressive drought (PD). Coefficient of non-photochemical quenching in steady-state (qN_Lss), coefficient of photochemical quenching in steady-state estimate of the fraction of open PSII reaction centers PSIIopen / (PSIIopen+ PSIIclosed) (qP_Lss), and fluorescence decline ratio in steady-state (Rfd_Lss) under field capacity conditions (FC) (A, B and C) and under progressive drought (PD) (D. E and F). Violet triangles indicated the moment of foliar application.*

Table IX.2. *Content of free amino acids (pmol g^{-1}) [average± Standard error (SE)] in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field water capacity (FC) or progressive drought (PD).of Arabidopsis seedlings untreated (Control) or treated with three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown under 80% field water capacity (FC) or progressive drought (PD) after the first (32 DAS) foliar application.*

	C		GT		BF		ST	
	FC	PD	FC	PD	FC	PD	FC	PD
AcGlu	2.1±0.6	3.4±0.9	2.8±0.7	4.1±1.0	7.2±0.9	4.8±1.2	2.2±0.4	2.1±0.6
Cys	18.7±1.9	4.7±1.9	3.4±0.8	8.2±1.3	5.6±0.9	17.9±2.6	8.6±2.8	18.7±1.9
Leu	5.1±1.3	9.1±2.1	12.4±2.6	8.7±2.4	39.1±15.3	14.0±1.2	6.6±0.5	5.1±1.3
Ile	7.7±1.3	10.4±2.9	15.2±1.4	12.6±2.5	52.1±10.7	14.7±1.8	9.1±2.2	7.7±1.3
Phe	42.0±5.7	35.1±4.6	24.1±1.5	28.5±4.4	33.1±2.8	17.4±1.4	18.3±3.7	42.0±5.7
Trp	5.0±1.0	4.5±1.4	5.3±0.9	6.6±1.0	10.8±0.9	9.2±1.4	4.1±0.4	5.0±1.0
GABA	50.6±11.9	50.1±12.0	26.7±1.2	15.8±3.0	39.5±11.6	25.3±2.1	31.4±8.4	50.6±11.9
BABA	15.7±4.5	8.5±2.3	9.7±1.8	14.7±3.2	30.7±9.6	16.8±6.0	43.6±32.4	15.7±4.5
Met	7.2±2.2	4.7±1.0	8.9±3.2	4.3±0.4	8.4±2.2	9.3±2.2	4.3±0.5	7.2±2.2
Val	59.0±15.3	49.5±12.6	55.8±8.1	57.7±15.5	191.5±8.7	180.0±41.9	38.1±8.2	59.0±15.3
Pro	62.3±13.0	62.1±10.4	58.8±12.9	65.2±16.2	190.1±14.1	104.6±25.6	45.9±15.7	62.3±13.0
Tyr	12.0±1.8	10.3±2.5	10.9±2.5	17.1±2.4	62.7±13.4	32.9±10.7	7.7±2.7	12.0±1.8
B-Ala	11.4±4.5	7.4±1.4	15.3±6.1	13.4±4.0	22.2±5.9	13.2±2.6	8.4±1.0	11.4±4.5
Ala	3.8±1.7	2.8±0.7	5.7±2.9	9.2±2.4	35.7±2.7	1.2±0.2	2.9±1.8	3.8±1.7
Hpr	3.7±1.2	2.8±0.5	4.1±1.4	3.1±1.3	7.3±0.5	6.3±2.7	1.7±1.0	3.7±1.2
Thr	253.5±35.1	363.4±38.3	964.7±110.4	909.5±162.2	1,706.9±317.4	688.9±159.1	343.7±72.6	253.5±35.1
Gly	78.8±4.6	48.8±7.9	157.4±38.5	45.6±4.7	89.4±17.6	27.9±7.6	31.2±6.3	78.8±4.6
Glu	1,456.1±201.2	1,021.8±172.2	2,806.4±368.9	823.1±195.1	2,243.4±137.5	1,877.0±245.7	1,013.2±194.8	1,456.1±201.2
Gln	1,327.0±250.0	1,627.2±429.8	1,212.4±164.4	1,761.7±212.0	2,620.2±765.7	2,535.4±88.0	1,884.4±288.8	1,327.0±250.0
Ser	171.9±21.7	266.0±75.1	676.4±78.5	355.9±75.2	1303.3±40.3	423.5±98.4	279.3±80.0	171.9±21.7
Asn	490.0±125.4	518.1±78.0	323.8±14.0	242.6±76.4	1,627.5±188.9	452.7±174.2	1,057.7±65.8	490.0±125.4
Cit	102.2±25.2	166.5±46.0	462.8±201.4	67.7±19.4	364.5±57.4	229.6±15.4	46.0±9.1	102.2±25.2
Asp	626.4±189.1	421.0±111.0	819.3±231.3	211.5±87.7	7,449.6±927.8	592.7±132.3	339.9±36.2	626.4±189.1
HomoArg	182.4±16.5	236.0±61.5	220.6±54.3	302.4±36.2	494.2±96.8	225.8±74.8	262.7±45.7	182.4±16.5
Arg	225.5±36.0	88.1±0.6	247.7±60.9	157.2±22.0	262.8±77.6	179.6±41.8	221.8±52.4	225.5±36.0
His	16.1±2.9	8.6±1.9	20.1±1.4	3.7±1.1	48.4±1.6	11.4±4.4	3.7±1.7	16.1±2.9
Lys	9.2±0.9	35.3±15.8	115.0±20.0	12.1±4.6	109.7±1.4	62.1±24.8	24.3±1.9	9.2±0.9
Orn	3.6±1.0	4.3±0.8	28.3±9.4	1.9±0.5	9.4±2.8	3.5±1.2	4.9±1.8	3.6±1.0
AcOrn	1.7±0.7	1.0±0.1	2.7±0.7	1.3±0.4	3.2±1.2	1.6±0.8	1.7±1.0	1.7±0.7
SAH	0.4±0.1	0.6±0.1	0.22±0.02	0.2±0.1	0.9±0.2	0.5±0.3	0.3±0.2	0.4±0.1

Table IX.3 *Content of free amino acids (pmol g^{-1}) [average± Standard error (SE)] in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field water capacity (FC) or progressive drought (PD).of Arabidopsis seedlings untreated (Control) or treated with three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown under 80% field water capacity (FC) or progressive drought (PD) after second (39 DAS) foliar application..*

	C		GT		BF		ST	
	FC	PD	FC	PD	FC	PD	FC	PD
AcGlu	4.6±0.8	1.7±0.9	0.8±0.4	0.9±0.3	1.5±0.6	1.1±0.4	0.6±0.2	0.8±0.3
Cys	10.0±2.0	12.3±1.5	2.9±0.5	15.1±3.0	2.6±0.4	3.4±0.8	4.5±1.7	5.9±1.2
Leu	12.7±4.1	9.8±3.6	5.0±2.1	6.1±1.8	6.0±2.0	31.8±4.6	35.8±13.5	8.3±2.6
Ile	20.1±5.9	11.1±3.9	5.6±2.4	12.9±1.1	13.5±1.7	48.5±9.6	54.3±18.8	6.3±0.8
Phe	41.0±8.9	12.3±6.4	7.8±2.3	6.9±1.2	7.6±2.2	33.6±6.4	40.2±14.9	8.1±1.9
Trp	6.4±0.4	4.7±2.1	2.1±0.9	2.8±0.2	2.9±0.8	5.6±1.0	7.3±2.7	3.2±0.5
GABA	10.1±1.1	11.1±4.2	9.6±2.8	6.1±1.3	9.6±3.0	17.1±2.2	14.0±4.9	29.2±12.5
BABA	16.4±3.5	3.0±0.7	3.0±2.2	17.1±6.4	15.5±5.5	116.2±8.9	75.3±27.5	10.4±2.5
Met	5.5±1.2	3.2±0.9	2.4±0.9	2.5±0.3	3.2±1.4	3.0±0.9	2.8±0.8	2.0±0.3
Val	68.7±2.2	37.7±8.8	24.8±5.9	37.4±5.9	41.0±10.1	140.1±5.1	128.7±52.2	33.6±9.7
Pro	85.2±6.1	27.7±4.1	25.3±10.1	32.3±5.7	43.9±8.0	134.8±8.2	95.0±36.5	25.4±4.7
Tyr	11.0±1.2	9.8±2.9	5.1±1.9	7.3±2.1	8.4±3.6	36.5±13.9	54.4±16.9	7.8±2.3
B-Ala	17.5±3.6	7.5±2.6	3.9±1.6	5.4±1.0	5.9±2.0	5.6±1.6	6.8±2.2	6.3±0.5
Ala	5.1±1.1	0.3±0.2	0.9±0.2	8.7±2.5	10.4±4.1	24.5±2.3	13.8±4.8	4.5±2.5
Hpr	5.7±1.2	1.4±0.3	0.6±0.2	2.6±0.2	3.3±1.1	10.7±1.5	7.6±2.7	1.8±0.2
Thr	574.9±43.9	166.9±22.6	70.9±10.8	342.2±64.5	323.4±83.7	629.6±12.3	534.7±223.4	434.7±112.4
Gly	142.6±37.0	8.0±1.5	13.5±1.8	6.8±1.0	15.5±2.0	46.5±6.5	40.6±15.3	12.8±1.7
Glu	1,339.9±56.5	312.3±73.7	340.6±134.5	211.8±31.4	1,141.8±152.8	1,890.9±219.2	2,794.2±1059.6	1,335.8±321.5
Gln	1,778.2±84.6	374.4±94.9	359.0±87.8	689.7±129.7	1,186.5±98.7	1,093.7±39.5	1,085.1±440.6	639.1±54.2
Ser	352.2±51.2	118.2±8.7	93.5±36.9	263.4±42.9	322.3±121.1	714.2±51.8	967.2±386.2	362.2±107.7
Asn	555.8±60.5	84.6±5.5	142.4±25.4	486.6±23.5	856.6±51.3	791.9±66.5	537.8±193.7	543.5±58.9
Cit	151.6±28.7	26.1±5.7	20.2±7.1	64.0±6.0	106.6±3.6	203.6±16.4	92.6±36.5	51.4±7.8
Asp	556.1±97.1	193.4±78.1	268.9±75.6	1,645.2±216.5	328.2±28.1	3,463.0±315.0	2,817.4±1029.9	548.0±65.4
HomoArg	312.0±35.5	44.7±10.4	39.8±5.1	149.0±27.3	155.4±25.4	197.4±10.2	211.6±93.5	134.0±18.9
Arg	132.9±14.1	39.7±10.0	28.5±4.8	45.0±15.0	81.6±6.6	149.9±15.2	162.5±65.1	39.4±11.1
His	10.6±3.2	2.1±0.2	2.5±1.1	12.3±1.6	6.0±2.0	24.2±2.4	24.1±11.5	10.2±1.6
Lys	22.4±4.0	4.9±0.9	9.0±2.7	27.5±5.8	18.4±5.9	371.0±89.8	561.6±215.7	26.3±9.7
Orn	3.0±0.1	2.9±1.9	1.5±0.4	1.2±0.5	2.2±1.1	19.8±2.7	24.3±10.4	0.7±0.1
AcOrn	2.6±1.3	0.3±0.1	0.3±0.1	0.5±0.2	1.3±0.4	1.3±0.7	2.0±0.6	0.5±0.3
SAH	0.5±0.1	0.1±0.1	0.12±0.04	1.4±0.3	0.3±0.2	1.3±0.2	0.6±0.3	1.1±0.4

Table IX.4. *Content of organic acid (pmol mg^{-1}) [average± Standard error (SE)] in Arabidopsis seedlings untreated (C) or treated with three commercial biostimulants [Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] under field water capacity (FC) or progressive drought (PD).of Arabidopsis seedlings untreated (Control) or treated with three commercial biostimulants [(Greetnal (GT), Basofoliar (BF), or SoilExpert (ST)] grown under 80% field water capacity (FC, upper panel) or progressive drought (PD, lower panel).*

FC

		Mal	Acet	Cit	Fum
C	**32 DAS**	3,463.4 ± 1,427.6	5,439.2 ± 3,979.5	4,053.6 ± 3,060.2	5,610.0 ± 1,144.7
GT		2,415.5 ± 459.6	5,443.4 ± 3,975.5	3,589.2 ± 468.3	5,914.1 ± 804.2
BF		3,137.4 ± 439.6	12,347.4 ± 3,545.6	3,186.1 ± 2,684.1	3,367.8 ± 1,612.4
ST		4,579.4 ± 755.0	5,983.2 ± 1,661.0	9,645.3 ± 1,767.6	13,239.4 ± 1,247.3
C	**39 DAS**	6,004.6 ± 2,343.1	8,150.7 ± 7,278.0	9,493.5 ± 5,427.7	12,611.5 ± 5,748.8
GT		3,293.7 ± 1,058.9	5,839.2 ± 1,982.5	5,394.3 ± 2,987.1	8,836.6 ± 3,515.3
BF		6,146.2 ± 6,003.5	12,131.3 ± 6,026.9	4,114.3 ± 1,962.6	3,166.6 ± 868.3
ST		3,433.8 ± 753.5	5,368.9 ± 3,863.3	7,383.9 ± 4,350.5	8,207.9 ± 4,712.4

PD

		Mal	Acet	Cit	Fum
C	**32 DAS**	2,690.6 ± 392.5	5,070.9 ± 2,440.0	3,620.8 ± 1,968.1	6,610.7 ± 791.0
GT		2,831.2 ± 1,005.5	4,356.0 ± 2,770.3	3,936.8 ± 627.5	4,771.0 ± 1,836.1
BF		2,339.0 ± 698.8	5,712.6 ± 6,265.2	3,093.5 ± 1,156.2	3,282.4 ± 705.3
ST		2,814.2 ± 769.6	7,746.3 ± 4,306.3	4,665.0 ± 1,875.6	6,191.3 ± 1,848.6
C	**39 DAS**	4,579.4 ± 755.0	5,983.2 ± 1,661.0	9,645.3 ± 1,767.6	13,239.4 ± 1,247.3
GT		5,290.3 ± 2,743.1	6,998.7 ± 4,108.5	7,735.4 ± 5,289.9	11,714.8 ± 5,438.7
BF		7,035.8 ± 699.1	12,166.7 ± 4,970.5	7,146.2 ± 1,425.8	10,735.9 ± 923.4
ST		4,423.2 ± 1,574.6	7,647.0 ± 1,033.0	8,869.8 ± 4,843.8	7,146.0 ± 2,571.5