Investigation of the mode of action of a plant extract against drought stress conditions

Cristina Sudiro1*, Federico Guglielmi1, Marie Hochart1, Andrea Ertani2, Serenella Nardi2, Biancamaria Senizza3, Begoña Miras-Moreno3, Luigi Lucini3, Adriano Altissimo1
1 LANDLAB srl, Via Quintarello 12/A, 36050 Quinto Vicentino (VI) - ITALY
2 Padua University, Agripolis - Viale dell'università, 16, 35020 Legnaro (PD) – ITALY
3 DISTAS - Department for Sustainable food process, Università Cattolica del Sacro Cuore, Via Emilia parmense 84, 29122 Piacenza - ITALY.
*correspondant author: c.sudiro@landlab.net

The biostimulant panorama is multifaceted and in continuous evolution, with many new products in the market, and the need of a more robust definition of the criteria for the demonstration of their efficacy. In particular, the need to investigate the mode of action of new and existing products through different technologies is strongly pursued in both the scientific and the regulatory community.

In the frame of the EU's LIFE Programme (LIFE18 ENV/NL/000043), the mode of action of a new plant extract (Plants for Plants 4-Vita) was studied. The application of product improved the response of treated tomato plants to drought stress. Beside the phenotypic results, it significantly and distinctively shaped the metabolomic profile of the crop, both under stress and at rewatering. In more detail, the treatment elicited a broad reprogramming of plant biochemical processes, as provided by both unsupervised hierarchical clustering and confirmed by supervised OPLS-DA modelling. Differential metabolites were interpreted using the PlantCyc omic viewer pathway tool, to point out the biosynthetic processes being modulated by the biostimulant. Under drought stress, nitrogen-containing metabolites (including glucosinolates and polyamide conjugates) and phytoalexins were elicited by the treatment, whereas phenylpropanoids were down-accumulated. Also the phytohormone crosstalk network was modulated by the biostimulant, particularly regarding brassinosteroids and ascorbate signalling.

Plants for Plants 4-Vita decreased oxydative stress in plants

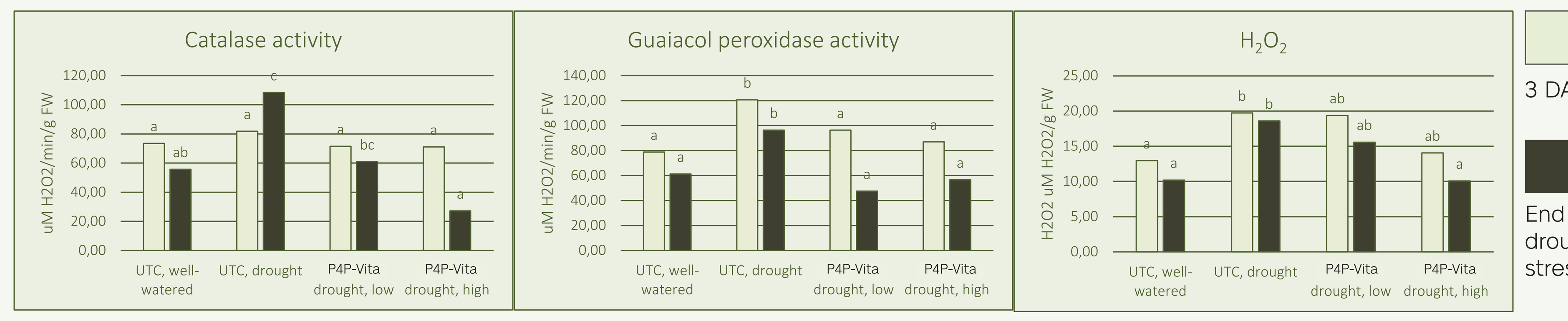
Plants for Plants 4-Vita was applied on sugarbeet seedlings, twice with 7 days interval. Irrigation was stopped the day after the second application. At the peak of drought stress, the plants were recovered for 14 days.

Effect on biomass

Entry	3 DAS		end of drought stress		14 DAR	
	FB shoot	FB root	FB shoot	FB root	FB shoot	FB root
UTC, 100%P	5,57 ab	1,96 a	4,15 ab	3,94 a	4,76 a	4,76 a
UTC, -30%P	5,13 a	1,55 a	3,46 a	3,79 a	4,18 a	4,81 a
P4P-Vita, dosage 1, -30%P	6,83 b	2,11 a	3,47 a	3,32 a	4,17 a	5,90 c
P4P-Vita, dosahe 2, -30%P	5,81 ab	2,03 a	5,23 b	3,95 a	6,19 b	6,51 c

Although after the re-watering, the shoot did not recover, the effect on the root, with high dosage Plants for Plants 4-Vita is clear. In fact, no statistical significant difference can be found between the positive control and this entry, implying that the photosynthetic machinery, necessary for the production of the photosyntate for biomass accumulation was not impacted irreversibly.

Effect on the redox status of the plants



The treatment with Plants for Plants 4-Vita decreased H_2O_2 content, thus decreasing oxidative stress. In fact, the enzymes for ROS detoxification (catalase and guaiacol peroxidase) where not as active as the UTC in drought conditions.

Plants for Plants 4-Vita improved tomato plants tolerance to heavy drought stress

At 3-4 leaves stage, tomato plants were treated twice with Plants for Plants 4-Vita with 7 days interval. The last irrigation was performed the day after the second treatment, and then the irrigation stopped until reaching maximum drought stress of the plants. During this time, the positive control (well-watered) was irrigated as usual. After 3 days from the last irrigation (4 days from last treatment), metabolomic analysis was performed on sampled plants. At the peak of drought stress, plants were recovered. Two weeks after the end on the stress, plants were harvested, the shoots weighted and the roots surface analyzed.

Entry	4 DAT	5 DAT	6 DAT	7 DAT	4 DAR
UTC, drought	4,9 a	4,3 ab	3,6 a	2,8 a	3,8 a
P4P - Vita, drought	4,9 a	4,2 a	3,5 a	3,5 b	4,0 b
UTC, well-watered	5,0 a	5,0 c	5,0 b	5,0 c	5,0 b

Visual evaluationof the wilting (5 = healthy plants)

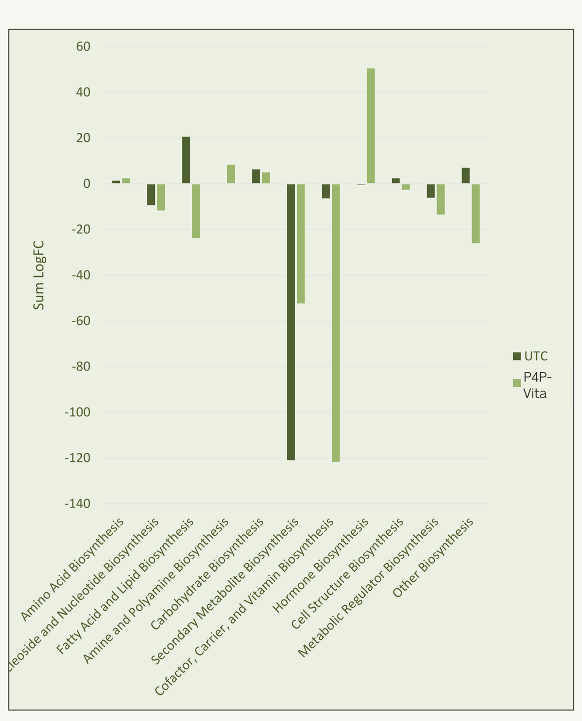
Entry	FB (g)	Flowers + Fruits	% roots
UTC, drought	26,0 a	4,6 a	5,1 a
P4P - Vita, drought	28,0 b	5,4 ab	5,9 a
UTC, well-watered	29,9 c	6,2 b	7,9 b

Final assessments

The effect of the frought stress was visible from 5 DAT (days after last treatement). Although at the beginning the level of wilting was similar between the treated and the negative control (UTC, drought), at the last day before recovery the treated plants showed less wilting compared to the negative control, suggesting that Plants for Plants 4-Vita application induced the activation of some tolerant mechanisms in the plants.

At the end of the recovery period, the treated plants showed an improved biomass compared to the negative control, as well as, as a trend, a higher amount of flowers and fruits, not different from the ones of the positive control (UTC, well-watered). Roots growth was instead impacted by drought stress in a similar way in both treated and untreated plants.

Plants for Plants 4-Vita induced changes in the metabolome



The drought stress induced a change in the metabolites content of the plant. However, this change was partially different when Plants for Plants 4-Vita was applied. In the tables and graphs the quantity of the metabolites found to be differentially expressed are expressed as LogFC compared to UTC well-watered. Thus the UTC in the tables is the UTC in drought stress (negative control). The differences, in particular, regard the compounds involved in fatty acid and lipid biosynthesis, secondary metabolite biosynthesis, cofactor, carrier and vitamin biosynthesis and hormone biosynthesis.

- Fatty acid and lipid biosynthesis: treated plants showed a general decrease in these compounds, while the UTC showed a increase. However, more compounds were involved in the treated plants compared to the UTC (21 VS 11), with an higher amount of compounds up- or down-regulated (Log FC > 2) than UTC (11 VS 7 – 10 VS 4)
- Secondary metabolite biosynthesis: treated plants showed less decrease in these compounds compared to the UTC, and more compounds involved in the change (95 VS 58). Of these, the 53 % was down-regulated in the treated plants, while in the UTC the 64% was down-regulated.
- Cofactor, carrier and vitamin biosynthesis: treated plants showed a dramatic decrease in these compounds, with 2/3 of the compounds involved down-regulated. The change in these compounds was less extreme in the UTC.
- Hormone biosynthesis: threated plants showed an increase in compounds involved in hormone biosynthesis, while this pathway was not particularly impacted by drought stress alone.

Fatty Acid and Lipid Biosynthesis

Compounds	Avg LogFC		Sum LogFC		Nr. of compounds LogFC>2		Nr. of compounds LogFC<2		Nr. of compounds modulated	
	UTC	P4P-Vita	UTC	P4P-Vita	UTC	P4P-Vita	UTC	P4P-Vita	UTC	P4P-Vita
Monogalactosyldiacylglycerol (MGDG)	0,08	-7,57	0,54	-52,99	0	1	0	4	0	5
Digalactosyldlacylglycerol (DGDG)	0,08	-10,47	0,08	-10,47			0	1	0	1
Phosphatidylglycerol	2,26	7,27	11,30	36,33	3	5	1	0	4	5

Treated plants showed a decrease in MGDG and DGDG and a huge increase in PG, while the untreated plants showed (lower) differences only in PG content. Regarding MGDG, there was an increase in the poli-unsaturated form (18.3; logFC 7,06 VS 0,05), that is usually correlated with higher drought stress tolerance (higher ROS scavenging and lower damage to cell lipids; Upchurch et al., 2008). Another feature of tolerance to drought stress is an increased DGDG:MGDG ratio (Upchurch et al., 2008). In the present experiment, although both decreased in treated plants, the decrease in MGDG was higher compared to DGDG, thus the DGDG:MGDG ratio appeared higher in the treated plants, implying a better tolerance to drought stress. The PG located in thylakoids play an essential role in the stabilization of PSI and PSII activity (Kobayashi et al., 2015), and in fact, the increase in this specific lipid type is much higher in the treated plants, suggesting that these plants are prepared to protect the photosynthetic apparatus. Also steroid lipids showed an increased level in treated plants (sum LogFC 25,9 VS 3,6). As steroids are involved in the activation of plant defense responses against various biotic and abiotic stresses (Pham et al., 1990), this change is an indication of an early signaling in the treated plants. As there was no visible sign of drought stress at 4 DAT (when the metabolomic analysis was performed), this early great change in the lipidome of the plants may be crucial for a development of tolerance during prolonged stress.

Secondary Metabolite Biosynthesis

Compounds	Avg LogFC		Sum LogFC		Nr. of compounds LogFC>2		Nr. of compounds LogFC<2		Nr. of compounds modulated	
	UTC	P4P-Vita	UTC	P4P-Vita	UTC	P4P-Vita	UTC	P4P-Vita	UTC	P4P-Vita
N containing compounds	-1,43	1,45	-51,45	52,21	4	13	14	13	18	26
Phenylpropanold Derivative Blosynthesis	-0,08	-1,78	-2,20	-46,15	10	8	7	12	17	20
Phytoalexin Biosynthesis	-1,30	3,65	-7,78	21,87	1	3	3	2	4	5
Terpenoid Biosynthesis	-1,12	-1,11	-71,61	-70,88	6	24	17	29	23	53

Treatments with Plants for Plants 4-Vita increased dramatically the level of N-containing compounds and compounds involved in phytoalexin biosynthesis, while decreasing the phenylpropanoid biosynthesis. The terpenoid biosynthesis was decreased in both untreated and treated plants, although the compounds involved in such changes were much higher in number in the treated plants (53 VS 23). Among the N-containing compounds the alkaloid class was highly induced, while L-Dehydroascorbate (L-DHA) was down-regulated. The phenylpropanoid class is formed by phenolic compounds (polyphenols, flavonoids, anthocyanins, ...). Both alkaloids and phenolic compounds have a strong antioxidant potential, however only the alkaloids pathway is induced, while the phenolic pathway seems to be downregulated (particularly for anthocyanins), suggesting a different modulation of the antioxidant potential due to Plants for Plants 4-Vita application. However, among alkaloids, chaconine and soladine are down-regulated, and this has been found to be a marker of drought stress tolerance potato. Furthermore, as there is a lower amount of L-DHA, a lower oxidative stress can be suggested in the treated plants. Among terpenoids compounds, xanthins shows a particular behaviour. In fact, adonixanthin and enochinone are down-regulated in treated plants, while canthaxanthin and antheraxanthin are upregulated. Adonixanthin and enochinone are in the biosynthetic pathway of the canthaxanthin, thus their down-regulation is consistent with an increase in the amount of canthaxantin. As a carotenoid, canthaxanthin protects the photosystems from oxidative stress, and this is consistent with the modulation of the lipidome shown in the previous paragraph. Also antheraxanthin is involved in photosystem protection, and in fact it is up-regulated.

CONCLUSIONS: Plants for Plants 4-Vita improved plants tolerance to drought stress by decreasing oxidative stress that is always associated with an osmotic stress. The activity of antioxidant enzymes is not increased during osmotic stress in treated plants, however H_2O_2 is lower, implying that there is either less H_2O_2 production or the activation of other antioxidant pathways. The early change in the metabolome induced by the treatment (higher protection of the photosystems, changes in the secondary metabolite biosynthesis, ...) gives further explanation on the mode of action of this product.

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