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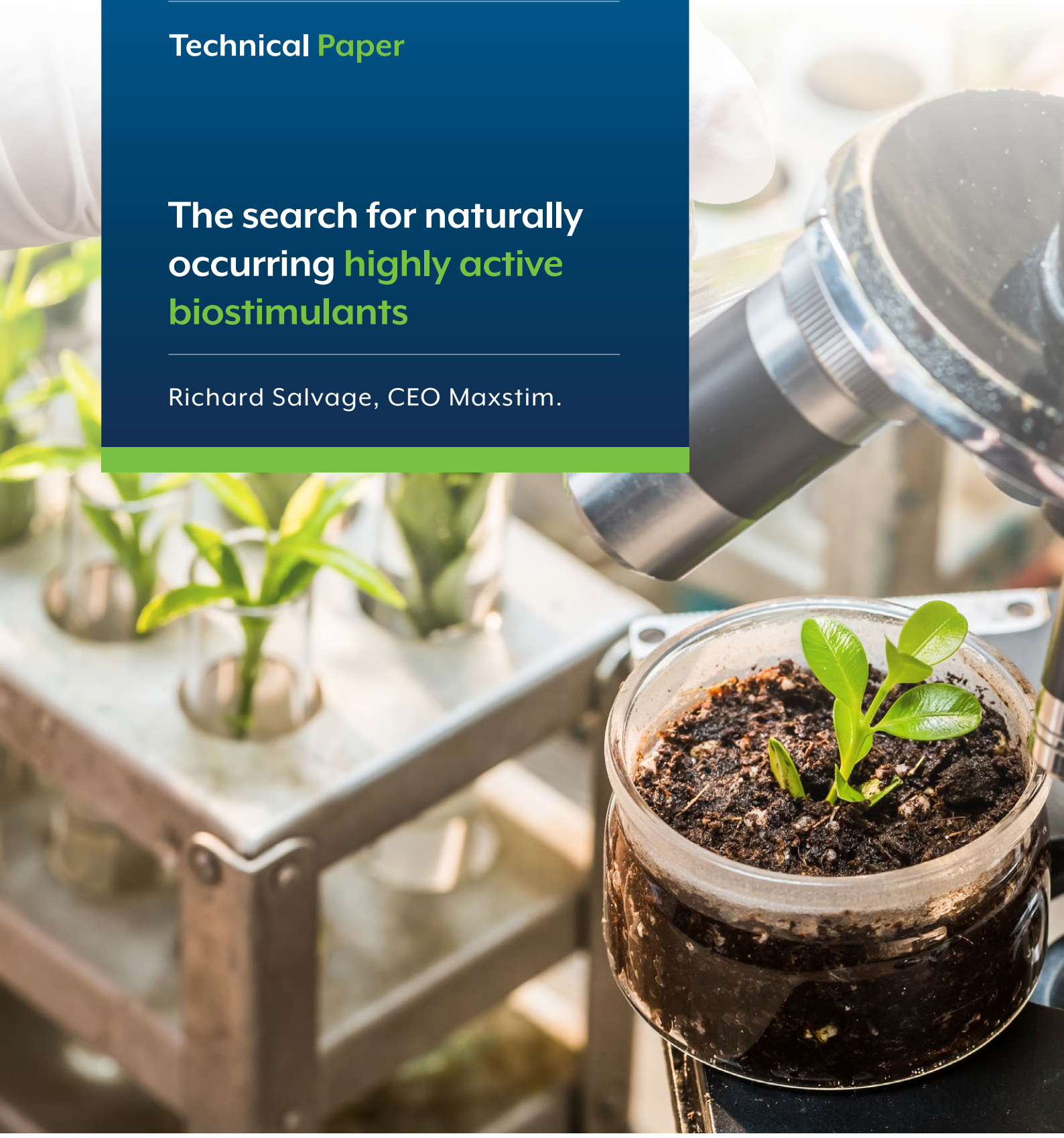
Technical Paper

The search for naturally occurring highly active biostimulants

Richard Salvage, CEO Maxstim.



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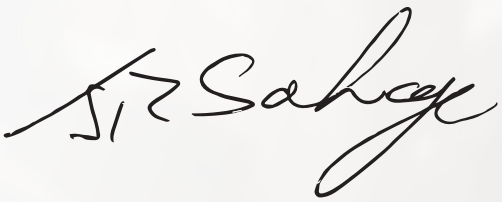
In the search for naturally occurring highly active biostimulants, Maxstim has sourced a unique range of bioflavonoids and polyphenols. We believe that bioflavonoids and polyphenols are important components in the armoury of any complex, state of the art biostimulant. This unique range of bioactive components have been patent protected and named Amphenox™.

Amphenox™ will be used to enhance Maxstim's future product range, heralding a new era of biostimulants and strengthening our intellectual property portfolio. Maxstim has identified production techniques that creates an abundance of bioactive compounds. The compounds of interest are polyphenols and more particularly the aglycones and glycosides of bioflavonoids.

Amphenox™ is rich in secondary metabolites that enable plants to turbocharge their immune systems and stimulate vital biochemical reactions such as growth, chlorophyll production, root development and stress management. In this technical paper I am sharing with you our process for evaluating Amphenox, the results and the methodology we use to create some of our products.



Richard Salvage,
CEO Maxstim.

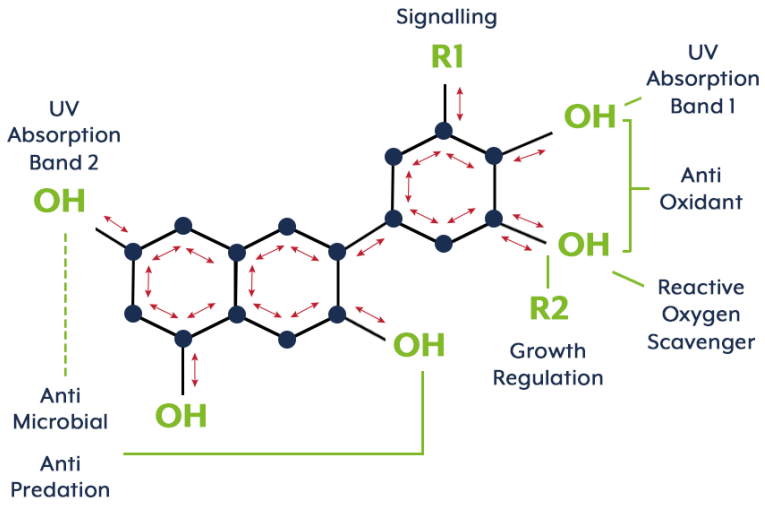
What are polyphenols and bioflavonoids?

Every living thing is made up of a huge number of essential chemicals all having different jobs to do in order to keep a plant functioning properly.

There are at least 8,000 different polyphenols which includes over 4,000 bioflavonoids.

Polyphenols are complex molecules but small enough to diffuse rapidly through cell membranes.

Bioflavonoids are very reactive and are responsible for colour in flowers, regulating growth, signalling to different parts of the plant and helping manage stress. They are some of the most versatile molecules in a plant's biochemistry. They are also the antioxidants for plants.



Bioflavonoids always have the same basic chemical makeup, but they have an amazing ability to change form and usage.

Depending on what a plant needs at any moment, Bioflavonoids can transform into a useful defence molecule and change into many different and very helpful tools.

When these bioflavonoid molecules join together in chains or polymers they can become antibiotic or probiotic in nature. So, this gives the plant the opportunity in some cases to protect itself from attack by bacteria, fungi and even insects.

The amounts of these specialist and versatile natural chemistries needed to help individual plants is very small. So, a small volume of bioflavonoids can go a long way in treating a large number of plants.

Amphenox™ has been assessed for its total polyphenol content and its antioxidant capacity. The results show that Amphenox™ has a significant level of polyphenols and bioflavonoids when compared to other known abundant polyphenol sources.

The polyphenol, bioflavonoid and antioxidant capacity of Amphenox™:

Amphenox™ Evaluation

Samples were prepared for investigation and were subjected to three rapid screening techniques to determine the total polyphenol content and assess antioxidant capacity by two separate electron tranport assays.

The total polyphenol assay employs Folin-ciocalteu reagent which is a highly coloured yellow molybdotungstophosphoric heteropolyanion reagent, where the molybdenum and tungsten are in the 6+ oxidation state. On reaction with polyphenol compounds these complexes are reduced and form a blue colour. The degree of the colour change is proportional to the polyphenol content.

When using the DPPH electron transfer assay, the highly coloured stable DPPH radical is introduced to an extract containing an antioxidant a reduction by either the antioxidant or radical species occurs. The absorption in turn decreases and the degree of the colour change is proportional to the antioxidant concentration.

In the second electron transfer assay, the stable oxidant ABTS ·+ cation is first generated by persulfate oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-6- sulfonic acid) (ABTS2-), this is then introduced to the extracted antioxidants which reduce the coloured cation by electron transfer with the degree of the colour change being proportional to the antioxidant concentration.

Methodology

Sample preparation:

Amphenox™ samples were centrifuged at 4500rpm for 10min at 4°C, before filtering through a 0.45µm syringe filter (Whatman).

Total Polyphenols:

Samples were diluted to an appropriate concentration in methanol and analysed in triplicate by adding in 20µl aliquots to individual wells in a 96well plate. 100µl of 2M Folin & Ciocalteu's reagent pre diluted 1:4 was then added to all wells and the plate shaken using a plate shaker for 4min. 75µl of 100g/l Sodium Carbonate solution was then added to all wells and the plate shaken for a further 1min using a plate shaker before an adhesive lid was applied.

The microplate was then incubated in the dark at room temperature for 2hours before reading at 750nm using a spectrophotometer.

DPPH:

Samples were diluted to an appropriate concentration in 80% methanol and analysed in triplicate by adding in 20µl aliquots to individual wells in a 96well plate. 280µl of 150µmol/l DPPH radical working solution was then added to all wells and an adhesive lid applied before the plate was shaken using a plate shaker set at 350±50rpm for 45min.

The microplate was then read at 515nm using a spectrophotometer. Antioxidant capacity was calculated as percentage of DPPH quenched relative to the reactivity of TROLOX as a standard under the same conditions.

ABTS:

Samples were diluted to an appropriate concentration in methanol and analysed in triplicate by adding in 20µl aliquots to individual wells in a 96well plate. 100µl of 2M Folin & Ciocalteu's reagent pre diluted 1:4 was then added to all wells and the plate shaken using a plate shaker for 4min. 75µl of 100g/l Sodium Carbonate solution was then added to all wells and the plate shaken for a further 1min using a plate shaker before an adhesive lid was applied.

The microplate was then incubated in the dark at room temperature for 2hours before reading at 750nm using a spectrophotometer.

LC-DAD-QTOF:

Polyphenolic analysis were performed on an Agilent 6510 QTOF mass spectrometer/ Agilent 1200 HPLC system equiped with a diode array detector (DAD). Separation was achieved using a Phenomenex Luna 5µ C18(2) 100Å LC (150mm x 2.0mm) column operated at 30°C. The mobile phase consisted of 100% deionised water containing 1% formic acid (mobile phase A) and 100% Acetonitrile containing 1% formic acid (mobile phase B). A gradient program was employed where %B ranged from 1% to 100% over a runtime of 81min.

The flow rate was held at 0.2ml/min and 5µl of each sample was injected.

Simultaneous monitoring of UV signals at 280, 320, 360 and 530nm was carried out. Accurate mass data was also collected in negative ESI mode over a mass range of 100-1000Da at a rate of 1.5spectra/s.

Results:

Sample	Lab	Dry Matter %	Total polyphenols mgGAEeq /g FW	DPPH µmol TROLOX eq /g FW	ABTS µmol TROLOX eq /g FW
AmphenoxTM	CAL195642	12.3	4.51	19.10	38.43
Lab standard	CAL191283	13.3	1.50	9.21	12.57

The atomic mass of each of the polyphenols and bioflavonoids was identified by Gas Chromatography Mass Spectroscopy, GCMS, techniques.

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AMA@ Oasis HLB SPE	LTD Orbitrap XL	10/12/2019 12:59:04

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+ p ESI Full ms
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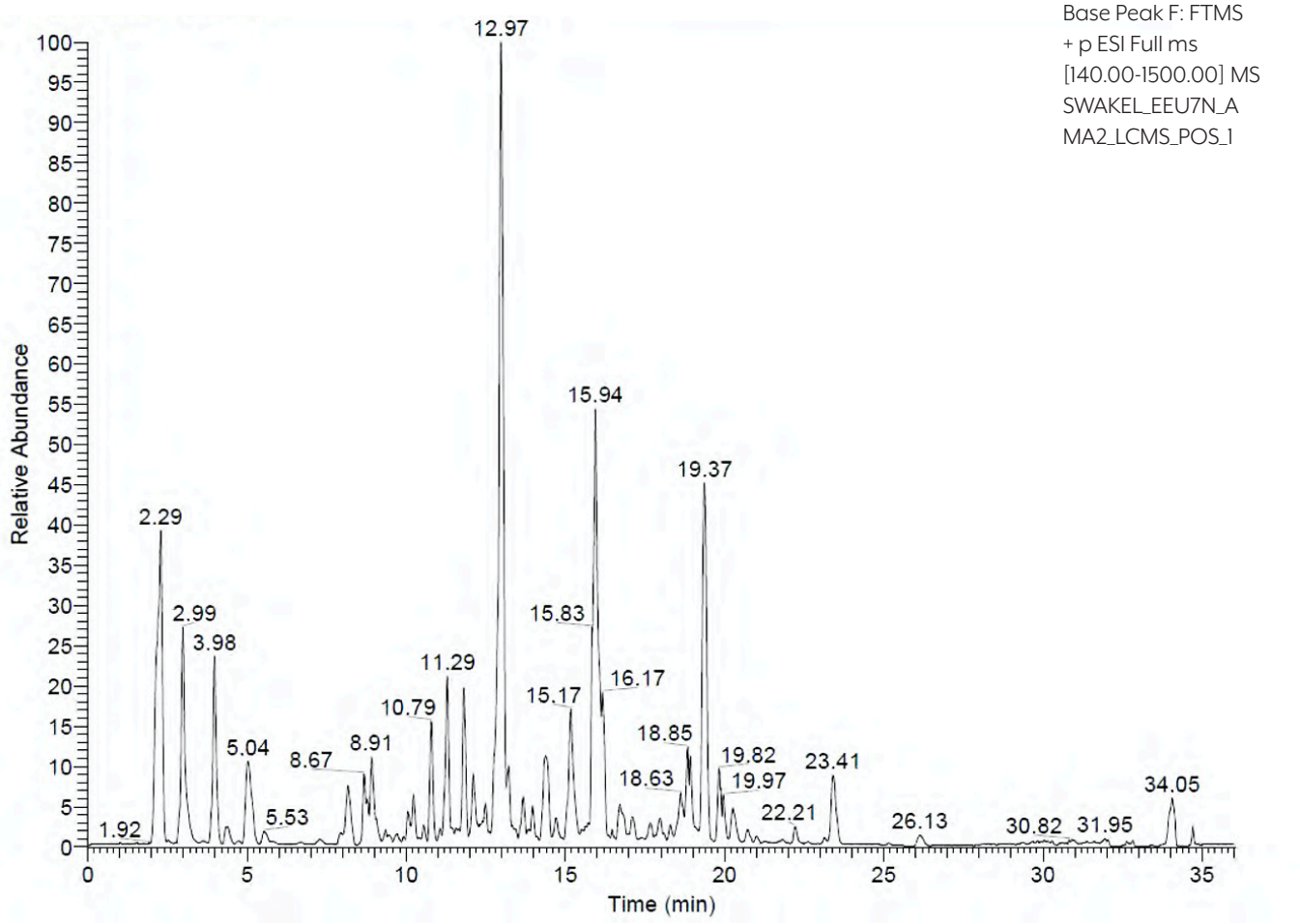
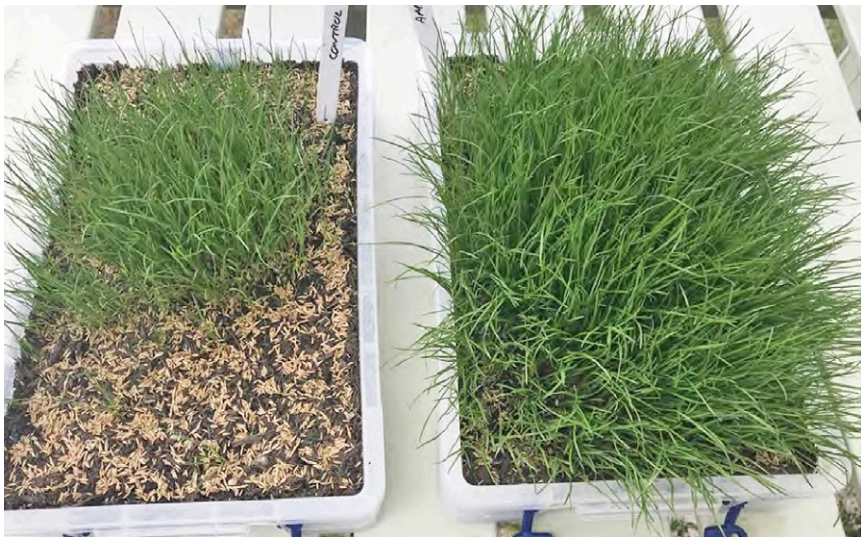


Amphenox™ efficacy.

The performance of Amphenox™ was assessed on both monocotyledon and dicotyledon plants using rye grass and lettuce as examples.

This first test was carried out on rye grass with a known weight of seed sown on each 4 litre container of compost. Amphenox™ was incorporated into the compost prior to the seed being sown.

Day 1



Day 12

Left: Control
Right: Ampenox

Day 30

Left: Control
Right: Ampenox

Amphenox™ on lettuce.

Various concentrations of Amphenox™ were applied to lettuce seeds.

Each pot contains 5 Butterhead lettuce seeds and was treated 4 times 7 days apart

Day 28 shows all four treatments way ahead of control pots placed here in the centre. Germination was more homogeneous and leaf development quicker and stronger

All four Amphenox treatments shown are way ahead of the control pots in the centre. (Row 3)

Day 40 showing the treated plants far more developed than the control pots placed on the far right.

Amphenox™ on rye grass.

A known amount of rye grass seeds were sown on five pots of compost. Various concentrations of Amphenox™ were applied to the seed and germinated plants at the rate of 100:1 in water each pot was treated 4 times at day 1,7,14 and 21 with 6ml of dilute product.

Day 28 shows a significant uplift on all Amphenox™ treated pots compared to control.

This leaf biomass data gathered on day 28 demonstrates that all Amphenox™ treatments are potent biostimulants increasing leaf biomass by at least 35%.

LEAF BIOMASS GRAMS						
	Pot 1	Pot 2	Pot 3	Pot 4	Pot 5	Total
Amphenox™ 1	10.6	13.5	13.9	13.0	12.3	63.3
Amphenox™ 2	9.8	13.6	15.7	13.4	11.8	65.3
Amphenox™ 3	13.1	13.5	13.7	15.6	13.7	69.6
Amphenox™ 4	11.3	12.7	14.1	12.0	14.7	64.8
Lab standard	8.1	8.9	13.9	9.6	6.5	46.2

Amphenox™ treatments increase leaf biomass by at least 35%

Amphenox™ combined with OSA to form Cynosa™.

Tested on Lettuce and Rye Grass

Amphenox™ was combined with ortho silicic acid, OSA, to assess the biostimulatory performance of the compound on an example of a monocotyledon plant, rye grass and an example of a dicotyledon plant, lettuce.

Five lettuce seeds were sown in each of five pots and their germination and development observed. Treatments were made at day 1, 7, 14, 21 with the control pots just receiving water.

Adding Amphenox™ to OSA speeded up germination and plant development compared to the control and compared to the OSA treated plants.

Day 40 shows both OSA and Amphenox™ ahead of control but the combination Cynosa™ (AMG4OSA) is considerably ahead of all the treatments and control thus demonstrating a synergistic effect.

Rye grass

Rye grass seed was sown on to pots and treated with OSA and Amphenox™ separately and combined as Cynosa™ and then compared to an untreated control.

Treatments were made at day 1, 7, 14, 21 with the control pots just receiving water.
The grass was harvested on day 28 and the leaf biomass measured and compared to the control

LEAF BIOMASS GRAMS						
	Pot 1	**Pot 2**	**Pot 3**	**Pot 4**	**Pot 5**	**Total**
Ortho Silicic Acid OSA	13.7	10.1	11.5	12.3	9.3	56.9
Cynosa™	12.9	14.0	11.2	16.5	14.5	68.1
Control	8.1	8.9	13.1	9.6	6.5	46.2

The OSA treated grass generated 23% more biomass than control. The combined Cynosa™ generated 47% more leaf biomass than control. Demonstrating a synergistic effect.

Cynosa™.

Cynosa™ is made up of a number of vital and very effective components, Amphenox™, orthosilicic acid (OSA) and surface active agents or surfactants. When combined they create a synergistic response and we use a very important natural process or phenomena known as Nanotechnology.

Nanotechnology in nature

Over millennia nature has perfected the art of biology at the nanoscale. Many of the workings of living cells occur at the nanoscale and rely on quantum and surface energy effects. A strand of DNA is around 2nm in diameter. A molecule of Chlorophyll consists of a porphyrin ring of 1.5 nm diameter with a 2nm.

Phytol tail. There are small cellular organisms that exist in the nanoscale range, such as viruses which are essentially living nanoparticles.

Some naturally occurring materials that we know to be beneficial to plants, such as silica, only have bioavailability and show bioactivity when presented as nanoparticles. Others such as nitrogen, potassium and phosphorous compounds are bioavailable both outside and inside the nanoscale range and can be readily assimilated as waterborne ions, which are not nanomaterials.

This suggests that the materials potentially available to plants can be allocated to one of three possible domains.

1) **Readily Bio-available Materials.** These include many water soluble elements, inorganic compounds and numerous organic compounds such as amino acids, polyphenols and terpenoids. They are sufficiently chemically reactive to exhibit bio-activity both outside, and inside the nanoscale range and their active species are capable of translocation to target cells within the plant.

2) **Bio-available, but only as Nanoparticles.** On conversion to nanoparticles these materials acquire enhanced availability and bioactivity and the ability to be translocated within the plant.

3) **Biologically Inert materials**. Do not exhibit bioavailability or bio-activity in any form.

Creating bioactive Cynosa™ nanoparticles

Producing nanomaterials by precipitation from solutions of soluble materials is our chosen method. The aggregation of orthosilicic acid molecules occurs once the Cynosa™ solution pH is reduced by diluting with water. An orthosilicic acid monomer has a theoretical size of around 0.6 nanometers and, as the solution is diluted, more of the monomer particles lose a hydrogen atom and acquire a single negative charge. As long as some uncharged particles are still present aggregation between them will occur, as will aggregation between charged and uncharged particles. As further aggregation occurs dimers, trimers, tetramers and larger particles are produced from the original monomers, most of which carry two negative charges. As their like charges repel each other they can only continue to aggregate with uncharged particles and once all the uncharged particles are used up all of the silicon is then present as negatively charged orthosilicic acid ions and so aggregation stops. These particles are then easily taken up by the plant to be transported around the plant to form thick dermal linings helping strengthen and protect the plant.

Cynosa™ combines all the latest technology that Maxstim Ltd has developed. Helping to strengthen plants and protect them against fungal diseases and Cynosa™ is perfect to act alongside Maxstim's biostimulants.

Maxstim's Amphenox™ is a patent protected, unique and abundant source of specific polyphenols and bioflavonoids.

Naturally sourced these organic polyphenols and bioflavonoids are a revolutionary addition to plant metabolic stimulation. When added to high quality biostimulants, Amphenox™, using nanotechnology, enhances their effects, boosting crop yield with a synergistic action improving key phenotypes and physiological properties.

Cynosa: a new silicon based biostimulant for arable crops, horticulture and sports turf.

Our understanding of silicon as an effective biostimulant led us to develop our new product Cynosa. It has been designed to specifically strengthen plants and protect them against fungal diseases. Rich in bioflavonoids, Cynosa is a perfect companion product to use alongside Maxstim's other biostimulants and will support crop development and growth throughout a plant's lifecycle.

If you would like to understand more please get in touch to request our technical report which demonstrates product efficacy following field testing.

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Technical Paper

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