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Title: Effects of humate supplemented with red seaweed (*Ahnfeltia tobuchiensis*) on germination and seedling vigour of maize

Chanyarat Paungfoo-Lonhienne[1], Thierry G. A. Lonhienne[2], Andrei Andreev[3], Lidya V. Zhiltsova[4], Sergey Kovalev[1], Alexey Belov[3], Igor Grebenyuk[3], Nikolai Kinaev[1], Evgeny Sagulenko[1]

[1]*Sustainable Organic Solutions Pty Ltd, Indooroopilly, QLD 4068, Australia,*

[2]*School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, QLD 4072, Australia.*

[3]*School of Engineering, Far Eastern Federal University (FEFU), 10, Ajax Str., Russky Island, Vladivostok, Russian Federation*

[4]*Pasific Scientific Researh Fisheries Center (TINRO-center), Shevchenko Alley, 4. Vladivostok, 690091, Russian Federation*

Corresponding author: Chanyarat Paungfoo-Lonhienne

Tel: +61-7-33469953 **Fax:** +61-7-33651699**; Email:** cplonhienne@sosbio.com.au

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Abstract

Humic substances (HS) are known as stimulators of seed germination and plant growth. Here we have examined the influence of HS obtained from the extract of a mixture of brown coal and industrial waste of red seaweed, *Ahnfeltia tobuchiensis* (HS-*Ahnfeltia*), on the germination of maize seeds. Seeds of *Zea mays* L. were immersed in HS-*Ahnfeltia* (treatment) or water (control) and germinated on moist filter paper in sealed Petri dishes. After 5 days of incubation at 28°C, the results showed that treatment with HS-*Ahnfeltia* promoted seedling growth. While no significant differences occurred in seed emergence, an important increase in root (36%) and shoot (54%) area as well as root (100%) and shoot (54%) dry weight was observed in treated seedlings, resulting in a 100% increase of the seedling vigour. These results suggest that HS-*Ahnfeltia* extract can be used as an effective agent to increase seedling vigour in crops.

Keywords: Humic substances, Maize, Red seaweed, Seed germination, Seedling vigour

Introduction

Humic substances (HS) are organic materials representing a major component of soil organic matter – humus. HS originates from plant, animal and microorganism decomposition, subsequently resulting in accumulation in soil, lakes, rivers, compost, sediments, peat and low-rank coals (Aiken et al., 1985). The major components of HS are humic and fulvic acids, their salts, and humin Aiken et al., 1985. HS is commonly extracted from brown coal by alkaline treatment although alternative methods do exist. Varying methods of extraction (Sire et al., 2009), coal source, and additives result in a wide range of physical and chemical properties of HS (Gogate et al., 2006; Lin and Yen, 1993; Mason et al., 2004). It has been shown that HS have beneficial effects on plants including: i) improved micronutrient availability (iron, copper, and zinc in particular) through formation of organo-metallic complexes that facilitates their absorption by plants (Chen et al., 2004); ii) improved water uptake efficiency (Morard et al., 2010); iii) improved abiotic stress tolerance (Canellas et al., 2015); and iv) a positive influence on root architecture, including the increase of lateral root development through induction of expression of the hormone auxin (Nardi et al., 2002; Trevisan, 2009). This emphasises why there is a growing interest in the use of HS as an agricultural biostimulant.

HS extracted from brown coal contains relatively small amounts of microelements necessary for plant growth. During remediation some products such as seaweeds are added to HS as a natural source of those elements. Red seaweed (*Ahnfeltia tobuchiensis*) is rich in mineral elements and nitrogen-containing substances (Kadnikova et al., 2014). *Ahnfeltia* is widely used in commercial applications as a major source of agar (McHugh, 2003), and the process generates a large volume of waste rich in minerals and polysaccharides.

Here our aim was to use this valuable nutrient source in the development of HS by adding *Ahnfeltia* waste during the process of HS extraction from brown coal. The results show that the use of HS-*Ahnfeltia* in seed germination of Maize has a positive effect on seedling vigour.

Results

Chemical compositions of HS-Ahnfeltia

The composition of HS-*Ahnfeltia* extract used for maize treatment was determined by chemical analysis. The major components of the extract are potassium (15%), phosphorus (1.7%), humic (2.1%) and fulvic (1.6%) acids (Table 1). The brown coal is the main source of humic and fulvic acids (the first 3 components shown in Table 1), however, it is very poor in microelement (below the detection limit). In contrast, the seaweed *Ahnfeltia* contains large amounts of microelements (Table 2) which are observed in HS-*Ahnfeltia* extract (Table 1).

The high level of potassium indicated in Tables 1 and 2 is related to the potassium hydroxide based extraction used here (see method). Piccolo et al (1993) have showed that concentrations of humic acid up to 0.5% increased seed germination and growth of seedlings without showing signs of growth inhibition. Based on this result, the HS-*Ahnfeltia* extract was diluted up to 20 times (5% HS-*Ahnfeltia* contains 0.1% humic acid) in this study. Our preliminary result with different concentrations (0.1, 2, 5%) of HS-*Ahnfeltia* showed that 5% has the best impact on maize seeding growth (data not shown), thus we repeated the experiment at 5%.

Seedling germination and vigour

After 5 days of incubation with 5% HS-*Ahnfeltia*, we observed a significant increase in root and shoot area of germinated maize seeds (Figure 1A). The average root area of HS-*Ahnfeltia* treated seeds (117.7±7.4 mm^2) was higher compared to untreated seeds (86.7±4.1 mm^2) (Figure 1B-1). The average shoot area also increased in treated seeds (19.3±1.4 mm^2), compared to control (12.5±0.9 mm^2) (Figure 1B-2). The root dry weight of HS-*Ahnfeltia* treated seeds (7.0±0.9 mg) was 2 times higher compared to control seeds (3.5±1.2 mg) (Figure 1B-3). The shoots dry weight also increased in treated seeds (4.7±0.3 mg) compared to control (3.1±0.5 mg) (Figure 1B-4). No significant changes were found in the seed germination (Figure 1C-1). Based on these results the vigour index was calculated giving a value of 250.8±4.2 for treated seeds and 125.2±8.7 for control seeds (Figure 1C-2). The results show that the treatment with HS-*Ahnfeltia* leads to a 100% increase of the seedling vigour of *Zea mays* L.

Discussion

Both beneficial and suppressive effects of HS on seed germination and seedling vigour have been variably reported; highlighting the importance of the composition of the HS extract and the process used for its extraction (Piccolo et al., 1993). Our results show that the HS-*Ahnfeltia* treatment of maize seeds appears to be highly efficient in promoting the seedling vigour of Maize, which is in agreement with other studies reporting that algal extracts can increase seed germination and vigour (El-Sheekh and El-Saied, 2000; Demir et al., 2006;

Carvalho et al., 2013). This could be attributed to the supplementation of mineral elements and nitrogen-containing substances in HS-*Ahnfeltia*.

In conclusion, the results of this study show that HS-*Ahnfeltia* extract is a potentially valuable product for increasing seed emergence and seedling vigour. It represents a sustainable system whereby natural waste products from seaweeds can be recycled into a quality organic fertiliser for use as an agricultural biostimulant. Further investigation needs to be undertaken to determine the *Ahnfeltia*-derived substances that are promoting the seedling vigour and the optimal HS*:Ahnfeltia* ratio, and to test HS-*Ahnfeltia* with different plant types in order to assess the generality of its positive effect.

Materials and Methods

Seed material

The cultivar of maize (*Zea mays* L.) used in this study was the variety PAC735, provided by Advanta Seeds. Seeds were stored in the dark in a cold room at 5°C in a resealable bag until required.

Humic and seaweed extract preparation

Brown coal was obtained from Shkotovo coal deposit (Far East Region of Russia), and *Ahnfeltia* from the Baklan Bay (Vladivostok, Russia). The seaweed was incubated in 4% KOH for 24 hours and subsequently mixed with brown coal (5:1). The mixture of brown coal and *Ahnfeltia* was treated using ultrasound cavitation technology (Andreev et al., 2015; Andreev et al., 2011) to extract the HS.

Seed treatment

Seeds were immersed in 5% aqueous HS-*Ahnfeltia* extract solution (w/v) or distilled water (control) for 5 minutes before placed, equidistant, on a sterilized 9-cm Petri dish containing filter paper (Whatman #1). The petri dishes received 6 mL of sterile water and were incubated in a growth cabinet (28°C, 16/8 h day/night, 400 µmol m^{-2} s^{-1}) for 5 days. Each petri dish contained 25 seeds, and each treatment consisted of 6 replicate petri dishes. The petri dishes were randomly rearranged in the growth cabinet daily. The experiment was repeated twice independently.

Analysis of seedling germination and vigour

The shoot and root areas were measured by the ImageJ 1.48v program (Schneider et al., 2012). For dry weight analysis, the roots and shoots were separated and dried at 60°C for 3 days to get a constant dry weight. % Emergence and vigour index were calculated using the following formula:

$$\% \text{ Emergence} = \frac{Number of emerged seedlings}{Number of seeds sown} x100$$

Vigour index = Germination% × Seedling dry weight (Abdul-Baki and Anderson, 1973)

Statistical analyses

Statistical analyses were performed using Student's *t* test (GraphPad Prism4, San Diego) to test for significant differences in seed germination and vigour of HS-*Ahnfeltia* treated *versus* control seedlings.

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Table 1. Chemical analysis of HS-*Ahnfeltia* extract

Analysis	Unit	HS-*Ahnfeltia*
Total humic substances	%	3.8
Fulvic acid	%	1.6
Humic acid	%	2.1
Nitrogen	%	0.1
Aluminium	mg/L	54
Boron	mg/L	88
Calcium	%	<0.01
Cobalt	mg/L	0.3
Copper	mg/L	<1
Iron	mg/L	99
Magnesium	%	<0.01
Manganese	mg/L	1
Molybdenum	mg/L	0.9
Phosphorus	%	1.7
Potassium	%	15
Sodium	%	0.15

Table 2. Chemical analysis of *Ahnfeltia*

Analysis	Unit	*Ahnfeltia*
Nitrogen	%	3.45
Calcium	%	0.63
Cobalt	µg/g	0.97
Iron	µg/g	108.6
Manganese	µg/g	11.8
Molybdenum	µg/g	0.9
Phosphorus	%	0.071
Potassium	%	5.3
Sodium	%	2.0

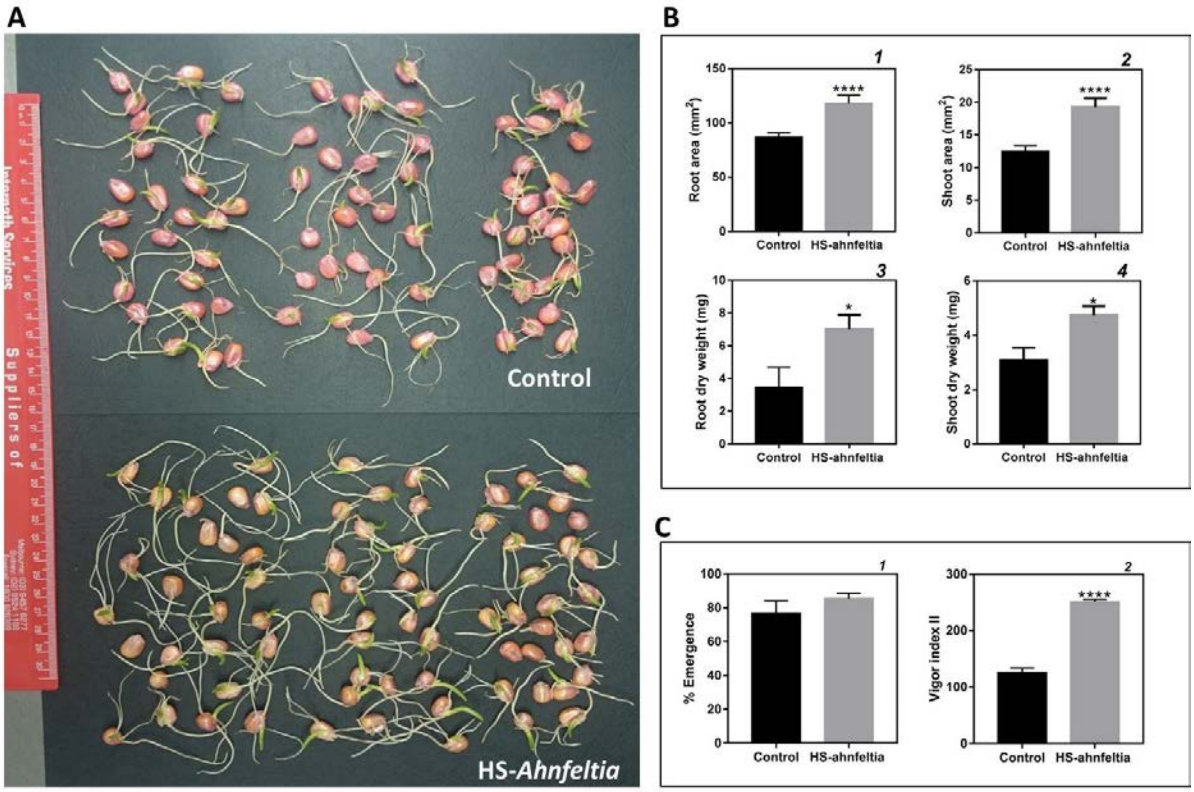

Figure 1. Effect of HS-*Ahnfeltia* extract on seed germination and vigour of maize (PAC735). (A) Germination after 5 days incubation of untreated seeds of maize incubated with water (top panel) or treated with 5% HS-*Ahnfeltia* solution (bottom panel). (B) Analysis of root and shoot area and dry weight. (C) Seed germination rate and seedling vigour index. Error bars denote standard error of the mean. Asterisks above bars indicate significant differences from the control: *, $P<0.05$; ****, $P<0.0001$ (Student's t test).