An integrated plant nutrition system (IPNS) for corn and cannabis in the Mid-Atlantic USA

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ABSTRACT

Agroecosystem and cycling loops are open when considering the reutilization of inputs in farming areas. Non-renewable resources have been transformed or relocated from the air, water and land into the system and are flowing out as wastes rather than reusable, recyclable resources. Therefore, current trends in agriculture have moved towards more sustainable cultivation systems with higher efficiency of input use, since mineral nutrient losses due to runoff, leaching, erosion and gas emissions are leading to environmental degradation. A huge variety of materials can serve as a crop nutrient supply and they can be derived from different resources. The integrated plant nutrition system (IPNS) thrives tailoring plant nutrition and soil fertility management, taking advantage of the conjunctive and harmonious use of inorganic, organic and biological resources. We hypothesize that the synergetic effects of the combination of humic acid HA + biofertilizer will improve plant agronomic outcomes when comparing the application of each product alone. We initiated this project conducting a greenhouse study and field experiments evaluating the effects of an IPNS on corn. Posteriorly, the positive results in terms of corn biomass increasing, led to another greenhouse study addressing cannabis (*Cannabis sativa* L.) due its valuable biomass as an end/selling product.

The greenhouse studies evaluated the effects of commercial synthetic fertilizer, HA, compost/manure teas and bioinoculant as inorganic, organic and biological resources, respectively, and their synergy on corn and cannabis early development under a period of water deficit stress. Generally, for both studies, when compared to the control values, the use of HA, biofertilizers and the integration of both substances generated significantly greater early season plant height, chlorophyll content and photosynthetic efficiency.

The three-year field trial investigated the effects of nitrogen (N) fertilizer, HA, compost/manure teas and bioinoculant as inorganic, organic and biological resources, respectively and their synergy on corn growth. The individual and integrated application of HA and biofertilizer generally influenced corn development, to varying degrees. In 2017, corn height, NDVI, greenness and vigor were sensitive to the application of these biostimulants in different magnitudes and growth stages, however grain yield and nutrient content were not affected. In combined studies from 2018 and 2019 corn height was not impacted by biostimulant application but NDVI, photosynthetic efficiency, greenness and vigor were affected at different doses and corn growth stages. Only one treatment integrating HA + biofertilizer led to increased grain yield.

In sum, these studies provided evidence that the individual and combined application of HA and biofertilizer can positively influence corn and cannabis growth most likely due to their plant biostimulant effects. However, the current study cannot conclusively affirm that the integrated use of HA and biofertilizers following the IPNS is

a superior practice than the application of each compound individually and further studies should be conducted to validate these findings.

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GENERAL AUDIENCE ABSTRACT

The status of the corn (*Zea mays* L.) demand in Virginia and the Mid-Atlantic region is currently in deficit regarding the production in this area. This demand is exceeding supply by approximately 150%, then increasing feed grain yield and consequently production in the region can be simultaneously beneficial to crop farmers, end-users and the entire food supply chain. Furthermore, the consumer market is becoming more aware about sustainable practices for food production, which encourages producers to adopt agricultural practices that can minimize negative environmental impacts. This scenario enforces the scientific community's responsibility to test and develop environmental-friendly methods able to increase fertilization efficiency, decreasing the use in synthetic inputs but maintaining yield. The integrated plant nutrition system (IPNS) implements the combined and harmonious use of inorganic, organic and biological resources to take advantage of the potential synergetic effects. We conducted greenhouse studies and field experiments evaluating the effects of an IPNS on corn, and posteriorly based on the preliminary results obtained with corn, a greenhouse study addressing cannabis (*Cannabis sativa* L.) was also carried out.

The greenhouse studies evaluated the effects of commercial synthetic fertilizer, humic acid (HA), compost/manure teas and bioinoculant as inorganic, organic and

biological resources, respectively, and their synergy on corn and cannabis growth under a period of drought. Generally, for both studies, when comparing to the control values, the use of HA, biofertilizers and the integration of both compounds generated significantly greater early season plant height and photosynthesis measurements.

The three-year field trial investigated the effects of nitrogen (N) fertilizer, HA, compost/manure teas and bioinoculant as inorganic, organic and biological resources, respectively and their synergy on corn growth. The individual and combined application of HA and biofertilizer generally influenced corn development, to varying degrees. In 2017, corn height, vegetation index, greenness and vigor were sensitive to the application of these biostimulants in different magnitudes and growth stages, however grain yield and nutrient content were not affected. In combined studies from 2018 and 2019 corn height was not impacted by biostimulant application but vegetation index, photosynthetic efficiency, greenness and vigor were affected at different doses and corn growth stages. Only one treatment combining HA + biofertilizer led to increased grain yield.

In sum, these studies provided evidence that the individual and combined application of HA and biofertilizer can positively influence corn and cannabis growth most likely due their plant biostimulant effects. Even though, the current study cannot affirm that the combined use of HA and biofertilizers following the IPNS is a better practice than the application of each compound individually, this practice can be a more sustainable alternative to fit in the conventional farming scene.

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ATTRIBUTIONS

Chapter 1: The Synergistic Effects of Humic Substances and Biofertilizers on Plant Development and Microbial Activity

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Chapter 2: Corn Response to an Integrated Plant Nutrition System (IPNS) with Humic Acid and Biofertilizers.

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Chapter 3: The Effects of an Integrated Plant Nutrition System (IPNS) on Cannabis Sativa Development.

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Chapter 4: An Integrated Plant Nutrition Systems (IPNS) for corn in the Mid-Atlantic USA.

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Chapter 1: The Synergistic Effects of Humic Substances and Biofertilizers on Plant Development and Microbial Activity

ABSTRACT

Agroecosystem and ecological cycling loops are open when considering the reutilization of inputs applied in farming areas. Non-renewable resources have been transformed or relocated from the air, water and land into the system and are flowing out as wastes rather than reusable, recyclable resources. This current environmental situation is promoting the development of methods able to optimize nutrient cycling, minimize use of external inputs, and maximize input use efficiency. This review strives to enhance our understanding of the conjunctive use of humic acid (HA) and biofertilizers. The biostimulant effects of each of these compounds are shown in the literature. Thus, we hypothesize that the combined application of HA and biofertilizers can promote synergy between both compounds and potentially more efficacy. The effects promoted by using HA plus biofertilizers on plants and microbes are very interconnected, so sometimes these effects can be confounded. For instance, the root elongation promoted by HA might increase hyphal fungi colonization. Therefore, this review was divided in three sections: responses of plants, fungi and bacteria. The findings indicate that the source and application rate of humic substances will have a strong impact on whether or not plant growth and microbial activity significantly improved. The microbial species and plant type also influence the response to humic substances. The prospects of the conjunctive use of humic substances and biofertilizers to stimulate plant development and microbial activity in agricultural systems are theoretically substantial.

Keywords: biostimulants, humic acid, biofertilizer, plant growth, microbial activity

INTRODUCTION

Soil is a fundamental requirement for food, feed, fuel and fiber production as it provides plants with support, water and nutrients. Even though mineral, organic and biological sources are present in soils, external applications known as fertilizers are necessary to improve plant development. Therefore, fertilization is an essential practice to enhance soil fertility, increase crop productivity and support agricultural intensification [1]. Plant nutrient resources are essential for agricultural intensification in the world's increasing demand for food and fiber [2]. A huge variety of materials can serve as sources of plant nutrients. These can be synthetic, natural, recycled wastes or a range of biological products including compost teas and microbial inoculants, which may increase the plant nutrient uptake.

The current status of agriculture has been influenced by several global trends as: environmental stewardship, population pressure, land constraints and agricultural policies [3] and relevance of each one is increasing. Thereby, the challenge for the near future is to sustainably maximize crop productivity [4, 5]. In this sense, reaching economic efficiency is the most pressing challenge for producers facing this reality in the agricultural segment. Thus, it is necessary to develop methods that optimize nutrient cycling, minimize use of external inputs, and maximize input use efficiency according to the conditions of each region [6].

The nature and characteristics of nutrient release on fertilizers derived from inorganic, organic and biological resources are different in terms of soil fertility and plant growth [7]. Therefore, the overall strategy to sustain high crop yields should include not only the addition of synthetic materials called plant food. But also, the integrated use of biological and organic nutrient resources might be a way to increase farming efficiency and minimize environmental impacts

[8]. This integrated approach recognizes that producers must nourish the soil, not just the plants, because a healthy soil will have major impact on nutrient availability, plant growth and agricultural sustainability. Thus, this is a preventive not curative technique, where soil fertility should be maintained instead drained from the soil. According to an FAO bulletin [9], an "integrated plant nutrition system (IPNS) or integrated nutrient management (INM) enables the adoption of the plant nutrition and soil fertility management in farming systems to site characteristics, taking advantage of the combined and harmonious use of mineral, organic and biofertilizer nutrient resources to serve the concurrent needs of food production and economic, environmental, and social viability."

The principle of IPNS is the balanced application of appropriate fertilizer to ensure that all the essential nutrients are maintained in the soil to match the nutrient availability to crop demand at any growth stage [10]. We investigated humic acid (HA) and biofertilizers as an organic and biological resource, respectively.

Humic substances are generally classified into HA, fulvic acid, and humin according to their pH solubility in water [11]. According to MacCarthy [12], they are heterogeneous mixture of organic materials formed by the decomposition of plant and animal residues. On the other hand, Lehmann and Kleber [13] argued that humic substances are not naturally occurring in soil. However, in both scenarios the use of humic products in agriculture could promote positive effects in a soil containing or not preexisting humic substances and in plant growth. The use of humic compounds present multiple environmentally friendly benefits alone and in combination with other products. The combined uses of organic and inorganic fertilizers not only optimize

each other's efficiency but can also gradually decrease the need for application of synthetic fertilizers [14]. The physicochemical activity and structure of humic substances can play an important role to support the sustainable agriculture demands because of their influence on improving soil quality and crop productivity [15, 16]. Humic acid have a high base exchange capacity which is important for soil stability. [17]. They also have a growth-stimulating effect and increase nutrient availability [18, 19]. These qualities mentioned previously are promising humic materials to be natural resources that might upgrade fertilizer efficiency.

Theoretically, biofertilizers are not fertilizers, which directly serve as plant food. A biofertilizer is a substance containing microbial cultures as fungi and bacteria in a carrier material, which can be used on seeds, plants, and soil to colonize the rhizosphere or the interior of the plants and increase nutrient availability in soil and consequently plant growth [20-22] Several studies have documented direct and indirect benefits of biofertilizers such as compost tea and bioinoculants. These compounds can increase agricultural sustainability in terms of significant enhancement of vegetative growth, yield and nutrient uptake by improving the physicochemical properties of the soil and increase of beneficial microbial populations for plants and soil fertility [23-25]. Moreover, solubilization of Phosphorus (P) or Potassium (K), uptake of Nitrogen (N) and multiplication of extraradical hyphae biomass are effects promoted by bioinoculants that might minimize negative impacts such as erosion and soil degradation [26, 27].

The potential benefits of humic substances and biofertilizers as plant-growth promoters and agents of nutrient acquisition, stress tolerance, and pathogen suppression are evident, and

substantial work has been done in this area. However, humic substances and biofertilizers are derived from multiple resources which can decrease the comparative level among research conducted in this topic. For instance, the comparison between HA derived from leonardite and lignite might not be fair. Studies have reported positive effects of the integrated use of humic substances + biofertilizers on yield of several plants such as: pineapple (*Ananas comosus*) [28], faba bean (*Vicia faba* L.) [29], tomato (*Solanum lycopersicum*) [30], wheat [31, 32], grapes (*Vitis vinifera* L) [33], garlic (*Allium sativum* L.) [34, 35], mungbean (*Vigna radiata* L.) [36], cucumber (*Cucumis sativus* L.) [37, 38], basil (*Ocimum basilicum* L.) [39], sorghum (*Sorghum bicolor* L.) [40], peach (*Prunus persica*) [41] and strawberry (*Fragaria ananassa*) [42]. Generally, these studies are not only showing that the combined use of HA plus biofertilizers positively affected yield but also presented the highest values when comparing to the alone application and/or control (no HA and biofertilizer). Also, they described the individual biostimulant effect as the main mechanism of action for HA and biofertilizers. Thus, the individual impact of HA and biofertilizers on primary and secondary metabolites plus nutrient uptake [43] can have important consequences on yield. Regarding the complementary effects of HA on biofertilizers and vice versa, HA stimulated microbial activity through ion exchange and metal complexing (chelating) systems [44, 45] and taking into account that HA might alter root exudation, consequently it can interfere with microorganism community in the rhizosphere [46, 47] Also, it increased the production of mycelium by mycorrhizal fungi [48]. Canellas, Balmori [24] speculated that the auxin-like action of HA might increase the colonization of Herbaspirillum seropedicae on corn via root-branching nodes. Similar results were found previously with the use of synthetic auxin 2,4-D [25]. Furthermore, in the same study, Canellas, Balmori [24] validated the synergetic effects in field trials, where corn yield was 48% and 45%

higher when humic acid and the bioinoculant (Herbaspirillum seropedicae) were applied in conjunction, compared with the individual use of HA and bioinoculant, respectively. Interestingly, the encapsulation of biofertilizer living cells in alginate beads enriched with HA enhanced shoot and root length on tomato and effectively protected the biofertilizer from the adverse condition of the soil [49]. Thus, the objective of this review is to have a better understanding of the effects of HA and biofertilizers on plant–microbial symbioses. These cooperative plant–microbial associations are very important components of nutrient cycling in agro-ecosystems and to enhance plant nutrient uptake [50, 51] Also, to clarify the effects of humic substances on plants, fungui and bacteria.

MATERIAL AND METHODS

To assess the effects of the conjunctive use of humic acid and biofertilizer on plant development and microbial activity, we conducted a search of the databases AGRICOLA and Google Scholar using a combination of search terms including: “humic” AND “acid” AND “biofertilizer” AND “plant” AND “growth” AND “microbial” AND “activity”. This search was designed to provide an unbiased selection of potential studies, rather than act as an exhaustive search for all studies in this area. A substantial number of articles related to at least one of these topics was found. However, we focused on the studies specifically addressing the integrated use of humic acid + biofertilizers. The variability of these articles in terms of source and dose of humic acid, plant species, microorganism strain and environment (laboratory, greenhouse and field trials) made it difficult to match up all categories. Therefore, this review was divided into three sections: responses of 1) plants, 2) fungi and 3) bacteria to humic acid, biofertilizers and/or humic acid + biofertilizer. Furthermore, to promote a fair equivalence of values when comparing

very different experimental conditions, we used the increment and /or decrement (difference) of each parameter in percent (%) units when comparing to the control values. Control was defined as the treatment not receiving humic acid, biofertilizer or both, in order to clarify the understanding of each category. We recognize that the effects promoted using humic acid and biofertilizers on plants, fungi and bacteria are very interconnected. Thus, these effects can be confounded. For instance, the root elongation promoted by humic acid could also increase the microbial colonization in the roots [52, 53].

RESULTS AND DISCUSSION

1.1 The effects of humic substances and biofertilizers on plants

Generally, the fourteen studies presented in Table 1 evaluate plant effects when HA and biofertilizer are applied in combination and when each compound is applied independently. Each study has its own specificities related to humic substances source, type of microbial strain included in the biofertilizer, fertilizer rates, plant species and plant parameters evaluated in response to the application of the treatments. In order, to correlate the data in this experiment's compilation (Table 1), we categorized the treatments presented in each study as 1) control, 2) humic substances, 3) biofertilizer and 4) humic substances + biofertilizer. Also, some studies did not have data to fit in all four categories, then we filtered out the studies containing at least control and humic substances + biofertilizer in order to evaluate the integrated use of both compounds.

The values in Table 1 showed that most of the studies presented positive differences or higher values when comparing the independent and combined application of humic substances

and biofertilizer against the control. In fact, among the total of 14 studies, 8 studies presented only positive responses, 6 presented negative and positive responses and none strictly showed negative responses when compared to controls (Table 1). Furthermore, the studies represented several yield components and all studies showed increase in productivity or plant growth when the optimal doses of humic substances + biofertilizer or either alone were applied in at least one of the parameters analyzed. Among the studies presenting only positive differences, the maximum contrast found in the studies were the following: Study 1 [54] presented 65%, Study 2 [55] 50%, Study 4 [28] 112%, Study 5 [29] 31%, Study 8 [35] 123%, Study 9 [36] 19%, Study 10 [38] 37%, Study 14 [42] 15% considering different plant species and parameters. The treatment promoting the highest positive difference when comparing to the control was humic substances + biofertilizers for all the cases. The expected mechanism for the biofertilizers were increased availability of soil nutrients, plant growth by production of phytohormones and enhanced disease control [56]. In addition, humic substances comprise a major part of organic matter influencing nutrient uptake and plant growth. Also they could improve microbial activity [57], which will be described in another section of this current study. Therefore, the combination of biofertilizers and humic substances might potentially improve plant productivity.

On the other hand, among the studies presenting mixed results on increases in yield were: Study 3 [58] 60% and -63%, Study 6 [30] 109% and -60%, Study 7 [33] 38% and -39%, Study 11 [39] 32% and -4%, Study 12 [40] 33% and -3% and Study 13 [41] 54% and -30% considering different plant species and parameter. In some studies, containing more than one parameter evaluated, the discrepancy of positive and negative effects appeared in different parts of the plant. In contrast to what happened with the studies containing only positive results, the studies

showing mixed effects was not consistent in terms of one specific treatment showing only positive or negative results. In these cases, HA, biofertilizer and the combination present negative values. Thus, the fact that the same treatment under different application rate led to extremely opposite results might infer that the improper application rate of these compounds could be an important factor to decrease performance. For instance, Study 3 [58] showed that the HA rate can also influence the interaction between plant and biofertilizers. It presented the highest root and shoot fresh weight values when *Glomus mosseae* + 300 mg/kg humic acid were applied. Nonetheless, the root and shoot weight values started to decrease progressively as the HA rates increased. The highest rate of humic substances and biofertilizer (*Glomus mosseae* + 3000 mg/kg humic) presented the lowest values even when compared with the control treatment which received only biofertilizer and no HA. Therefore, inappropriate humic substances rates might harm the positive effects of the conjunctive use of these compounds.

Table 1. Study 1: Effect on maize grain production (kg/ha) in field experiments. Control plants received 50 kg N/ha as urea. Treatments consisted of one foliar application (300 L/ha) of HA (20 mg/CL), Herbaspirillum seropedicae (log 109 cells/ml) and humic substances + H. seropedicae. Study 2: Dry-matter yield (g) of wheat. Study 3: Effect of increasing concentrations of HA on the growth of laurel plants in the natural soil inoculated with Glomus mosseae. Study 4: The effects of the integrated use of HA with two biofertilizers (*Burkholderia* sp. UENF 114111 and Burkholderia silvatlantica UENF 117111). Study 5: Grain yield in faba beans tested under the independent use of HA and in combination with Azotobacter. Study 6: Tomato biomass was tested under three substrates: commercial (control), (ii) vermicompost:soil (biofertilizer), and vermicompost:soil fortified with a solution of humates and H. seropedicae suspension. Study 7: Grape development evaluated with the application of HA, biofertilizer (Saccharomyces cerevisae) and two rates of N fertilizer. Study 8: Garlic growth and production tested under 12 different fertilization treatments including potassium humate and nitrogen biofertilizers alone and in combination. Study 9: Mungbean production tested under the application of HA and plant growth promoting rhizobacteria. Study 10: Cucumber production tested under several doses of HA and biofertilizer in combination and alone. Study 11: Basil growth tested under three fertilization practices: vermicompost, biofertilizer and HA. Study 12: The responses of sorghum development to the application of three different biofertilizer and combination with HA or alone. Study 13: Peach production tested under different rates of N fertilizer, HA and biofertilizer (Spirulina Platensis algae). Study 14: Strawberry yield tested under the combined and independent use of HA and biofertilizer.

Effects of the combination of humic and biozertilizers on plant/ biomass/ grain production.

Study 1: A combination of humic substances and Herbaspirillum seropedicae inoculation enhances the growth of maize - Canellas, Balmori et al. (2013) (Field trials)

Treatments	Maize grain yield (kg/ha)	Difference (%) ¥
Control	2600 c *	0
Humic substances	3042 b	17
Herbaspirillum seropedicae	3120 b	20
Humic substances + Herbaspirillum	4620 a	65

Study 2: Influence of sodium humate on the crop plants inoculated with bacteria of agricultural importance - Gaur and Bhardwaj (1971) (Greenhouse)

Treatments	Wheat grain yield (g)	Straw (g)	Straw + Grain (g)	Difference (%) (Grain + Straw)
Control	24.1 a	30 a	54.1 a	0
Na-humate	27.8 a	42 a	69.1 a	27.7
Azotobacter inoculation	24.6 a	32 a	56.6 a	4.6
Azotobacter + Na-humate	28.7 a	44.6 a	73.3 a	35.4
Bacillus inoculation	27.7 a	35 a	62.1 a	14.7
Bacillus + Na-humate	28.5 a	44 a	72.5 a	34

Bacillus + Azotobacter	27.2 a	39.3 a	66.5 a	22.9
Bacillus spp. + Azotobacter + Na-humate	32.6 a	49 a	81.6 a	50.8

Study 3: Influence of humic acids on laurel growth, associated rhizospheric microorganisms, and mycorrhizal fungi - Vallini, Pera et al. (1993) (Greenhouse)

Treatments	Laurel shoot fresh weigh (g)	Difference (%)	Laurel root fresh weigh (g)	Difference (%)
Glomus mosseae (no humic acid)	1.58 b	0	2.56 b	0
Glomus mosseae + 300 mg/kg humic acid	2.52 a	59.5	3.96 a	54.7
Glomus mosseae + 700 mg/kg humic acid	1.57 b	-0.6	2.40 b	-6.3
Glomus mosseae + 1500 mg/kg humic acid	1.64 b	3.8	2.52 b	-1.6
Glomus mosseae + 3000 mg/kg humic acid	0.91 c	-42.4	0.94 c	-63.3

Study 4: Growth promotion of pineapple'Vitória'by humic acids and Burkholderia spp. during acclimatization - Baldotto et al. (2010) (*In vitro* propagation)

Treatments	Pineapple shoot dry matter (g)	Pineapple root dry matter (g)	Shoot + root (g)	Difference (%) (Shoot + Root)
Control	0.34	0.07	0.41	0
Humic acid	0.38	0.07	0.45	9.8
Biofertilizer 1	0.62	0.10	0.72	75.6
Biofertilizer 2	0.66	0.10	0.76	85.4
Biofertilizer 1 + Humic Acid	0.76	0.11	0.87	112.2
Biofertilizer 2 + Humic Acid	0.61	0.11	0.72	75.6

Study 5: Effect of nitrogen, humic acid and biofertilization on productivity and quality of faba bean under saline condition - Bayoumi and Selim. (2012) (Field trials)

Treatments	Grain yield - Giza (g)	Difference (%)	Grain yield - Sakha (g)	Difference (%)
Control	46	0	32.2	0
Humic acid	54.4	18.3	33.8	5.0
Humic acid + Biofertilizer	60.1	30.7	35.5	10.2

Study 6: Substrate biofortification in combination with foliar sprays of plant growth promoting bacteria and humic substances boosts production of organic tomatoes - Olivares et al. (2015) (Greenhouse)

Treatments	Tomato shoot dry mass	Difference (%)	Tomato root dry mass	Difference (%)
Control	57.67 c	0	41.8 a	0.0
Biofertilizer (Vermcompost)	75.72 b	31.3	18.47 b	-55.8
Biofertilizer + Humic acid	120.3 a	108.6	16.82 b	-59.8

Study 7: Minimizing the quantity of mineral nitrogen fertilizers on grapevine by using humic acid, organic and biofertilizers - Eman et al. (2008) (Field trials)				
Treatments	Grape yield weight - 2005 (kg)	Difference (%)	Grape yield weight - 2006 (kg)	Difference (%)
Control (100% N mineral)	6.59 ab	0	14.76 ab	0
50% mineral N + Humic Acid	4.02 b	-39.0	16.44 a	11.4
50% mineral N + Humic Acid + Biofertilizer	9.07 a	37.6	19.02 a	28.9
Study 8: Effect of potassium humate, nitrogen, biofertilizer and molybdenum on growth and productivity of garlic (*Allium sativum* L.) - Mohsen et al. (2017) (Field trials)				
Treatments	Plant dry weight - Season 1 (g)	Difference (%)	Plant dry weight - Season 2 (g)	Difference (%)
Control	8.23 j	0	8.17 i	0
Potassium humate	11.98 g	45.6	10.55 g	29.1
Halex-2 (Biofertilizer)	14.78 ef	79.6	14.10 e	72.6
Potassium humate + Halex-2	18.33 c	122.7	17.79 c	117.7
Study 9: Integrated effects of humic acid and bio fertilizer on yield and phosphorus use efficiency in mungbean under rainfed condition - Sarwar et al. (2014) (Field trials)				
Treatments	Mungbean grain yield (t/ha)		Difference (%)	
Control	1.591		0	
Humic acid	1.78		7	
Humic acid + Plant growth promoting rhizobacteria	1.965		19	
Study 10: Use of humic acid and some biofertilizers to reduce nitrogen rates on cucumber (*Cucumis sativus* L.) in relation to vegetative growth, yield, and chemical composition - El-Shabrawy et al. (2010) (Field trials)				
Treatments	Cucumber yield 2007 (kg)	Difference (%)	Cucumber yield 2008 (kg)	Difference (%)
Control	0.93	0	0.95	0
Humic acid	1.16	24.7	0.99	4.2
Azobacter	0.96	3.2	0.97	2.1
Azospirillum	0.95	2.2	0.97	2.1
Humic acid + Azobacter	1.27	36.6	1.22	28.4
Humic acid + Azospirillum	1.2	29.0	1.18	24.2
Study 11: Vermicompost, plant growth promoting bacteria and humic acid can affect the growth and essence of basil (*Ocimumbasilicum* L.) - Befrozfar et al. (2013) (Field trials)				
Treatments	Basil shoot dry weight (kg/ha)		Difference (%)	

Control	1263 e	0
Humic acic 1 (soil drench)	1363 cde	7.9
Humic acid 2 (foliar)	1213 e	-4.0
Plant growth promoting bacteria	1400 cde	10.8
Humic acid 1 + Plant growth promoting bacteria	1663 c	31.7
Humic acid 2 + Plant growth promoting bacteria	1638 cd	29.7

Study 12: Synergetic effects of biofertilizers containing N-fixer, P and K solubilizers and humic substances on *Sorghum bicolor* productivity - Afifi et al. (2014) - (Field trials)

Treatments	Sorghum dry weight (g)	Difference (%)
Control	132.1 no	0
Humic Acid	128.2 p	-3.0
Azospirillium brasilense cells (Azo)	140 l	6.0
Bacillus circulans cells (Bc)	170 d	28.7
Bacillus megaterium cells (Bm)	130 op	-1.6
Azo + Bc + Bm	170 d	28.7
Humic acid + Azospirillium brasilense cells	166 e	25.7
Humic acid + Bacillus circulans cells	131 op	-0.8
Humic acid + Bacillus megaterium cells	155 gh	17.3
Humic acid + Azo + Bc + Bm	175 c	32.5

Study 13: Partial replacement of mineral N fertilizers by using humic acid and spirulina platensis algae biofertilizer in Florida Prince Peach orchards - El-Khawaga. (2011) - (Field trials)

Treatments	Peach yield/tree (kg) - 2010	Difference (%)	Peach yield/tree (kg) - 2011	Difference (%)
100 % inorganic N. (control)	20	0.0	21.7	0
90 % inorganic N + 40 ml Humic + 5 ml Biofertilizer	22.1	10.5	23.8	9.7
80 % inorganic N + 50 ml Humic + 10 ml Biofertilizer	24.3	21.5	25.4	17.1
70 % inorganic N + 60 ml Humic + 15 ml Biofertilizer	27	35.0	28.1	29.5
60 % inorganic N + 70 ml Humic + 20 ml Biofertilizer	29.4	47.0	30.5	40.6
50 % inorganic N + 80 ml Humic + 25 ml Biofertilizer	32.2	61.0	33.3	53.5

40 % inorganic N + 90 ml Humic + 30 ml Biofertilizer	14	-30.0	15.1	-30.4

Study 14: Effects of applying humic acid and bio-fertilizers on the qualities and yields of strawberry and soil agrochemical characters - Liu et al. (2015) (Greenhouse)	
Treatments	Strawberry yield in comparison to control values (%)
Control	0
Humic acid	10.8
Biofertilizer	7.7
Humic acid + Biofertilizer	14.7

* Means among columns, by study, followed by different letters are significantly different at *P* = .05. or 1. The studies without letter have not provided this statistical data.
¥ Difference when comparing to the control values in (%) units.

The fact that the 14 studies presented on Table 1 showed the humic substances, biofertilizer and humic + biofertilizer treatments promoting positive and negative effects, made it not very clear in terms of how beneficial is the application of these compounds. Therefore, we compiled the entire Table 1 in Figure 1, promoting a better visual comparison of the results found in these studies. We took into consideration all the data, including the studies evaluating more than one plant parameter and studies that did not presented all four categories. However, control and HA + biofertilizer were present in the whole list of studies. The Figure 1 confirmed the synergy of the integrated practice, where humic substances + biofertilizers presented the highest value on the stimulation of plant development (Figure 1). When averaging the values of each treatment in Table 1 and comparing to the control values, the results showed that humic substances, biofertilizer and humic substances + biofertilizers presented 11%, 16% and 29%, respectively, higher effects on plants (Figure 1). Indeed, Figure 1 is combining many different plant species, growth and yield parameters, sources and rates of humic substances and biofertilizers but there are not many studies related to this topic and it can be a

broad guidance. The independent use of humic substances on diverse species of plants have shown its influence on cell elongation [59], nutrient uptake [60], improved soil structure and water retention [61] and hormone-like effects [62]. Furthermore, studies have shown that numerous types of biofertilizers have the potential to act on plant development, as a result of different mechanisms, such as nitrogen fixation in legumes and non-legumes [63], solubilization of phosphates, micronutrient and minerals [64, 65], plant defence against biotic and/or abiotic stress [66] and stimulation of plant growth regulators like auxins, gibberellins, and cytokinin [67]. Thus, Figure 1 may represent one or more effects of these mechanisms caused by the application of humic substances, biofertilizers and/or both.

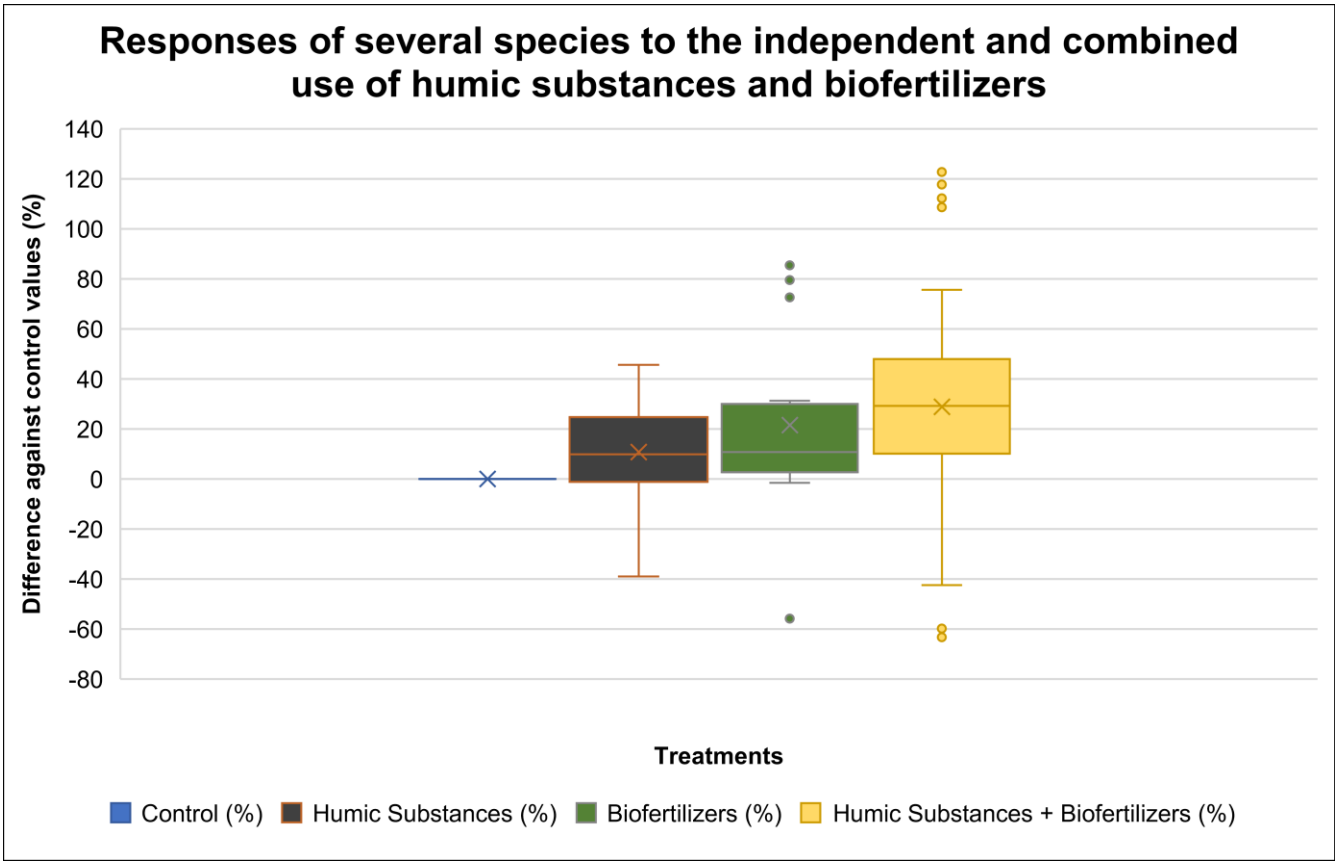

Figure 1. The compilation of results of 14 studies presenting the effects of the independent and integrated use of humic substances and biofertilizers on several plant species under different growing conditions.

1.2 The effects of humic substances and biofertilizers on fungi

The responses of fungal growth under increasing rates of humic substances are presented in Table 2, where Study 3 [58] was cited again. These studies, Study 15 [68], Study 16 [48] and Study 3 [58], were conducted in laboratory, greenhouse and open nursery environments, respectively. However, they have in common the fact that mycorrhizal development was measured when humic substances were applied. In this case, two studies (Study 15 and 16) showed positive results and Study 3 showed negative results in terms of mycorrhizal growth (Table 2). Study 15 presented the maximum of 88% and 80% increase of ectomycorrhizal growth in pH 7 and pH 4, respectively, when the optimal rates of fulvic acid were applied in agar

surface inoculated with *Pisolithus tinctorius*. According to Pettit [69], fulvic acid molecules are smaller than those of HA and they have more oxygen in their structure thereby increasing chemical reaction, even though both compounds derived from the same source. Then, in theory, the use of fulvic acid would be more efficient than HA due its higher contact area and capability to be adsorbed by plant leaf when HA size make it incapable. However, other important factors as application rate, environmental conditions, plant and microbial species can change the performance of these compounds. Study 16 tested only one humic substance application rate and it increased a mycelium growth in 158% using HA + biofertilizer compared with the control (only biofertilizer, no HA). The biodegradation of humic substances by fungi has been showed in the literature [70, 71]. Thus, a possible explanation for the mycorrhizal growth increment by humic substances in these two studies (15 and 16) is the fact that it can be used as a source of nutrients by the two species of fungi tested. Furthermore, as occurred with the plant parameters, the same treatment under different concentration promoted opposite results. On Study 16, the optimal humic substances rate under pH 4 resulted in 80% higher dry weight of Ectomycorrhizal fungi when comparing to control, but the greater dose of humic substances decrease it 37%. Also, in the same study Ectomycorrhizal fungus under pH 7 were not responsive to the application of 3200 ppm of humic substances (Table 2). Again, the humic substances doses appearing as an important factor on the performance of these compounds. Interestingly, Zhou and Banks [72] affirmed that the humic substances adsorption capacity of a fungi will depend on pH, ionic strength, concentration of metal ions and temperature of the environment.

The results on Study 3 implied that humic substances are harming fungal development. Differently than Study 15 and 16, it showed only negative effects on fungi growth. The control

(no humic substances) presented the highest value of hyphal length and as the humic substances rates increased, the hyphal values decreased progressively (Table 2). Previous study on the ectomycorrhiza of Douglas-fir showed that it was not affected by humic amendments [73]. In these cases where fungal development is not affected or showed negative hyphal growth, the plant is potentially losing the capability to increase nutrient uptake acting in synergy with fungi that could be rapidly growing and reaching more area. The results found in Study 3 partially agreed with those of Study 15 in which the application of the highest humic substances rate (3200 ppm) resulted in the lowest values of Ectomycorrhizal fungi. However, in Study 15 lower rates of humic substances enhanced ectomycorrhizal growth and it did not occur in Study 3. These studies show the extreme importance of HA rates in the interaction between microorganisms and plants. Thus, according to the different results obtained in these three studies (Table 2), it is important to emphasize that different humic substances rates, environments, plants and microorganisms might cause variable results.

The humic substances rates of studies 15, 16 and 3 were converted to the same unit (parts per million) and compared on Figure 2. Clearly, humic substances derived from different sources and concentrations can lead to positive, negative or no effects on fungi growth (Fig.2). The biofertilizer type, fungi strain and pH of the environment are also important factor in the interaction with humic substances.

Table 2. The effects of different doses of humic substances on mycorrhizal growth. Study 3: Effects of increasing concentrations of humic acid on hyphal length of Glomus mosseae. Study 15: Effects of fulvic acid and pH on dry weight of ectomycorrhizal fungus. Study 16: Effect of humic acid on mycorrhizal mycelium in agar substrate.

Effects of the combination of humic substances and biofertilizers on fungi development				
Study 3: Influence of humic acids on laurel growth associated rhizospheric microorganisms, and mycorrhizal fungi - Vallini, Pera et al. (1993)				
Dose of Humic acid (mg/kg)	Hyphal length (mm)		Difference (%) ¥	
Control	855		0.0	
25	495		-42.1	
50	515		-39.8	
100	553		-35.3	
200	350		-59.1	
400	137		-84.0	
800	317		-62.9	
3200	258		-69.8	
Study 15: Fulvic acid and the growth of the ectomycorrhizal fungus, Pisolithus tinctorius - Tan and Nopammornbodi (1979)				
Fulvic acid treatments	Dry weight (mg) of Ectomycorrhizal fungi (pH 7.0)	Difference (%) pH 7.0	Dry weight (mg) of Ectomycorrhizal fungi (pH 4.0)	Difference (%) pH 4.0
Control	57	0.0	30	0.0
320 (ppm)	65	14.0	27	-10.0
640 (ppm)	92	61.4	54	80.0
1600 (ppm)	107	87.7	33	10.0
3200 (ppm)	57	0.0	19	-36.7
Study 16: Hyphal growth and mycorrhiza formation by the arbuscular mycorrhizal fungus *Glomus claroideum* BEG 23 is stimilated by humic substances - Gryndler, Hrselová et al. (2005)				
Humic acid treatments	Length of mycelium (m/g)		Difference (%)	
Control	6.88 a *		0	
249 (mg/L)	17.78 b		158.4	

* Means among columns, by study, followed by different letters are significantly different at P = .05. Study 3 and 15 did not provide statistical information for this parameter.

¥ Difference when comparing to the control values in (%) units.

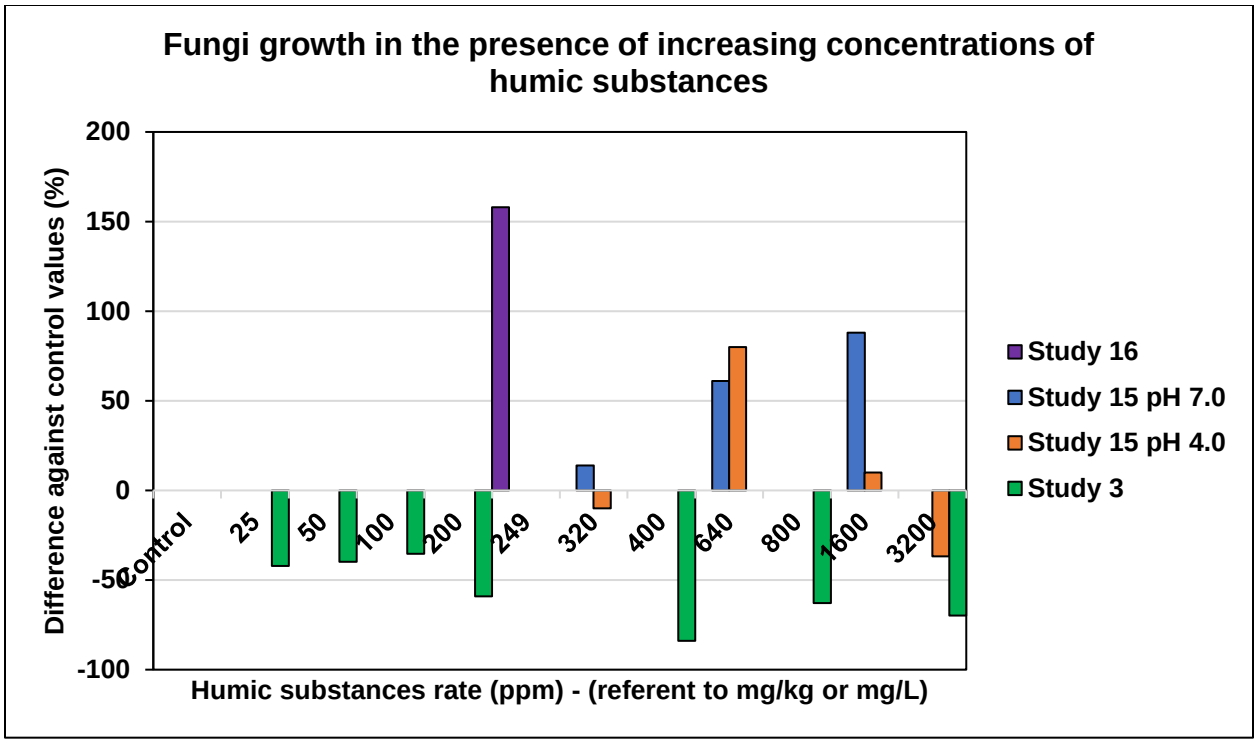

Figure 2. The responses of mycorrhizal growth to increasing application rates of humic substances presented in Study 3,15 and 16.

1.3 The effects of humic substances and biofertilizers on fungi

Many authors have reported that humified substances positively increased bacterial growth, activity and affected metabolic reactions [45, 74, 75]. Table 3 is a data compilation containing a vast number of different species of bacteria and evaluated the capability of bacteria grown under the addition of humic substances. Five studies were included in this data gathering: Study 2 [55], Study 12 [40], Study 17 [76], Study 18 [77] and Study 19 [78]. Study 2 and 12 were mentioned previously in the plant's effects section. In Study 2 the Rhizobium growth stimulated by HA is estimated according to the number of nodulations on dhaincha (*Sesbania aculeata*) and Study 12 used most probable number (MPN) technique to quantify the growth of Azospirillium brasilense, Bacillus circulans and Bacillus megaterium. In fact, most of the bacteria strains responded positively, and it can represent that humic substances contributed to

bacteria development (Table 3). The three bacteria strains tested in Study 12 responded positively to the application of humic substances, where none of them were incapable to grown under humic substances. Study 17 showed 108 soil and earthworm bacteria strains capable on growing under humic substances application and 55 being incapable. Study 2 and 18 presented 2 bacteria strains (one each) where humic substances showed to be beneficial for bacterial growth. Finally, Study 19 did not specify the exact number of strains, but several microorganisms enhanced growth after humic substances application. Using ^{15}N-labeled HA, Vaughan and Malcolm [79] found that HA was a source of N for several microbial species: *Bacillus megutarium*, *Pseudomonas jhorescens*, *Actinomyces glohisporus* and *Mycobacterium citreum*. According to Visser [80], HA, if added to selective media, could increase the growth of a wide range of taxonomic and functional groups of soil bacteria. It mostly confirmed what was found in Table 3. Also, the same author mentioned that a modification of microbial cellular activity and growth might be promoted by humic substances through their influence on cell membrane permeability and nutrient absorption [78]. This data compilation presented substantial diversity of bacteria responding positively in the presence of HA; then it might reinforce the hypothesis that humic substances increase microbial activity.

Table 3. Number of bacteria strains capable and incapable on growing under the application of humic substances. The parameters used to measure microbial growth were number of nodules (Study 2); most probable number (Study 12); optical density (Study 17); number of cells (Study 18); microbes per area (Study 19).

Study 2: Influence of sodium humate on the crop plants inoculated with bacteria of agricultural importance - Gaur and Bhardwaj (1971)					
Soil bacteria strain	Capable	Incapable	Bacteria (earthworm digestive tract)	Capable	Incapable
Rhizobium	1	0	Not applicable		
Study 12: Synergetic effects of biofertilizers containing N-fixer, P and K solubilizers and humic substances on Sorghum bicolor productivity - Afifi et al. (2014)					
Soil bacteria strain	Capable	Incapable	Bacteria (earthworm digestive tract)	Capable	Incapable
Azospirillium brasilense	1	0	Not applicable		
Bacillus circulans	1	0			
Bacillus megaterium	1	0			
Study 17: Effect of humic acids on the growth of bacteria - Tikhonov, Yakushev et al. (2010)					
Soil bacteria strain	Capable	Incapable	Bacteria (earthworm digestive tract)	Capable	Incapable
Aminobacter aminovorans	3	0	Acinetobacter spp.	4	0
Agromyces spp.	2	0	Aeromonas spp.	12	4
Arthrobacter spp.	2	2	Bacillus spp.	2	0
Bacillus spp.	16	8	Buttiauxella spp.	3	2
Kocuria palustris	3	5	Chryseobacterium spp.	2	0
Nocardioides spp.	2	3	Delftia acidovorans	1	4
Pseudomonas spp.	9	1	Microbacterium sp.	3	2
Rhodococcus spp.	1	0	Ochrobactrum grignonense	1	0
Sphingopyxis spp.	1	1	Paenibacillus sp.	1	0
Streptomyces spp.	1	3	Pseudomonas spp.	9	3
Microbacterium sp.	0	1	Shewanella sp.	0	1
Oxalobacter sp.	0	1	Rhodococcus sp.	0	1
Unidentified strains	16	8	Unidentified strains	21	5
Study 18: The effect of humic and fulvic acids on the growth and efficiency of nitrogen fixation of *Azotobacter chroococcum* - Bhardwaj and Gaur (1970)					
Soil bacteria strain	Capable	Incapable	Bacteria (earthworm digestive tract)	Capable	Incapable
Azotobacter chroococcum	1	0			
Study 19: Physiological action of humic substances on microbial cells - Visser (1985)					
Soil bacteria strain	Capable	Incapable	Bacteria (earthworm digestive tract)	Capable	Incapable
Amylolyc organims	Several	0	Not applicable		
Proteolytic organisms	Several	0			
Denitrifying organisms	Several	0			

The humic substances concentration rate was not considered in Table 3, therefore we simply assumed and counted the growth of bacteria when any rate of HA was added. Furthermore, Table 3 was basically enumerating the bacteria strains that can grow in conditions where humic substances are added; it is not a comparison to identify if the use of HA is better than to neglect its use. However, the studies cited in Table 3 provided information regarding the application or absence of humic substances, which was discussed in Table 4. There, the application rate was considered in order to conduct the comparison between the use or not of HA on bacterial development. In fact, the HA concentration or rate is extremely important as this review has shown previously in the plant and fungi interaction (Figures 2 and 3). It was not different when addressing the bacteria interaction with humic substances. Thereby, Table 4 included the studies mentioned in Table 3, but the humic substances concentrations were assessed and their effects compared. The optimal application of humic substances presented the maximum increment of 3900%, 1747%, 84%, 1567% and 100% in Study 2, 12, 17, 18 and 19 respectively. Puglisi, Fragoulis [44] reported the HA chelating effects on the increment of microbial activity. In most of the cases, the bacterial growth presented optimal humic substances rate. In other words, the bacterial growth was not directly proportional to the increment of humic substances concentration. In fact, we compared different bacterial strains and mainly methods to measure bacterial growth. Therefore, the magnitude of increase from one study to another is very high. Although, generally the application of humic substances increase microbial activity, where some rates promoted higher effects.

Table 4. Effects of humic or fulvic acid rates on bacteria development. Study 2: Effects of Na – Humate + Rhizobium on nodulation of dhaincha. Study 12: Effect of biofertilizers and humic substances on most probable number (MPN) of Azospirillum spp. and plate count of B. megaterium and B. circulans at 75 days. Study 17: Maximal specific growth rate of soil and earthworm intestinal bacteria in the nutrient medium with HAc (0.1 mg/ml) and without it. Study 18: Effects of sodium humate and fulvic acid on the growth of Azotobacter chroococcum. Study 19: Effects on molecular weight fraction of amylolytic, proteolytic and denitrifying microorganisms with Aldrich HA incorporated in organic soil at various concentrations.

Study 2: Influence of sodium humate on the crop plants inoculated with bacteria of agricultural importance - Gaur and Bhardwaj (1971)				
Treatments	Number of nodules on dhaincha			
	Uprooting II (growth stage)	Difference (%) ¥	Uprooting III (growth stage)	Difference (%)
Control (no humic)	15 *	0	50	0
Na-Humate + Rhizobium	600	3900	490	880

Study 12: Synergetic effects of biofertilizers containing N-fixer, P and K solubilizers and humic substances on Sorghum bicolor productivity - Afifi et al. (2014) - (Field Trials)						
Treatments	Most probable number (MPN) (×100000 cfu/g rhizosphere) after 75 days					
	Azospirillum	Difference (%)	B. circulans	Difference (%)	B. megaterium	Difference (%)
Control	4.5	0	10	0	5	0
Humic Acid	5.6	24.4	13	30	7	40
Azospirillium brasilense cells (Azo)	65.7	1360.0	15.1	51	22	340
Bacillus circulans cells (Bc)	36.6	713.3	54	440	11.1	122
Bacillus megaterium cells (Bm)	37	722.2	9	-10	77	1440
Azo + Bc + Bm	83.1	1746.7	56.1	461	90	1700
Humic acid + Azospirillium brasilense cells	79.5	1666.7	17.3	73	23	360
Humic acid + Bacillus circulans cells	37.1	724.4	57.5	475	12	140
Humic acid + Bacillus megaterium cells	3.9	-13.3	9.9	-1	87.7	1654
Humic acid + Azo + Bc + Bm	80	1677.8	56.1	461	86.6	1632

Study 17: Effect of humic acids on the growth of bacteria - Tikhonov, Yakushev et al. (2010)				
Humic acid concentration (mg/ml)	Maximum specific growth rate			
	Soil Bacteria	Difference (%)	Earthworm Intestinal Bacteria	Difference (%)
Control (no humic)	0.019	0	0.03	0

Humic acid (0.1 mg/ml)	0.035	84	0.045	50

Study 18: The effect of humic and fulvic acids on the growth and efficiency of nitrogen fixation of *Azotobacter chroococcum* - Bhardwaj and Gaur (1970)

Humic acid concentration (ppm)	Cell number 10000000/ml					
	Humate 1	Difference (%)	Humate 2	Difference (%)	Fulvic acid	Difference (%)
Control	2.4	0	2.4	0	2.4	0
20	4.5	88	4.3	79	6.1	154
100	8.3	245	8.3	246	12	400
200	21	775	20	733	25	942
300	36	1400	36	1400	37	1442
500	40	1567	40	1567	38	1483
700	34	1317	35	1358	38	1483
1000	25	942	25	942	28	1067
14000	21	775	20	733	20	733

Study 19: Physiological action of humic substances on microbial cells - Visser (1985)

Humic acid concentration (mg/L)	Number of microbes (log 10000000) per gram					
	Amylolyc	Difference (%)	Proteolyc	Difference (%)	Denitrifying	Difference (%)
10	4	0	11.5	0	4	0
20	5	25	11.8	2.6	5	25
50	6	50	12	4.3	5.5	37.5
100	7	75	12.2	6.1	6	50
500	8	100	12.5	8.7	6	50

*Studies 2 and 18 presented statistical differences between control and humic acid treatments, but information regarding multiple comparison analysis were not provided. Studies 12, 17,19 and did not provided statistical information for these parameters.
¥ Difference when comparing to the control values in (%) units.

The humic substances application rates from study 2, 12, 17,18 and 19 were converted to the same unit (parts per million) and compared in Figure 4. The studies evaluating only one rate of humic substances (Study 2, 12 and 17) showed that the application of humic substances increased bacterial growth when comparing to control where no humic substances were used. The studies testing more than one humic substances rates (Study 18 and 19) generally showed that bacterial biomass started to increase when the first doses of humic substances were applied, then the biomass reached the maximum value or plateau. Posteriorly, the humic substances concentration continued increasing but the bacterial biomass started to drop consistently. Again, it represented the importance of an appropriate application rate when HA is used as a biostimulant in combination with biofertilizer. Prakash and Rashid [81] tested various fractions of HA on marine phytoplankton development and they claimed that HA have different physiological effects according to the applied concentration, where the highest HA dose was not always correlated with the greater growth.

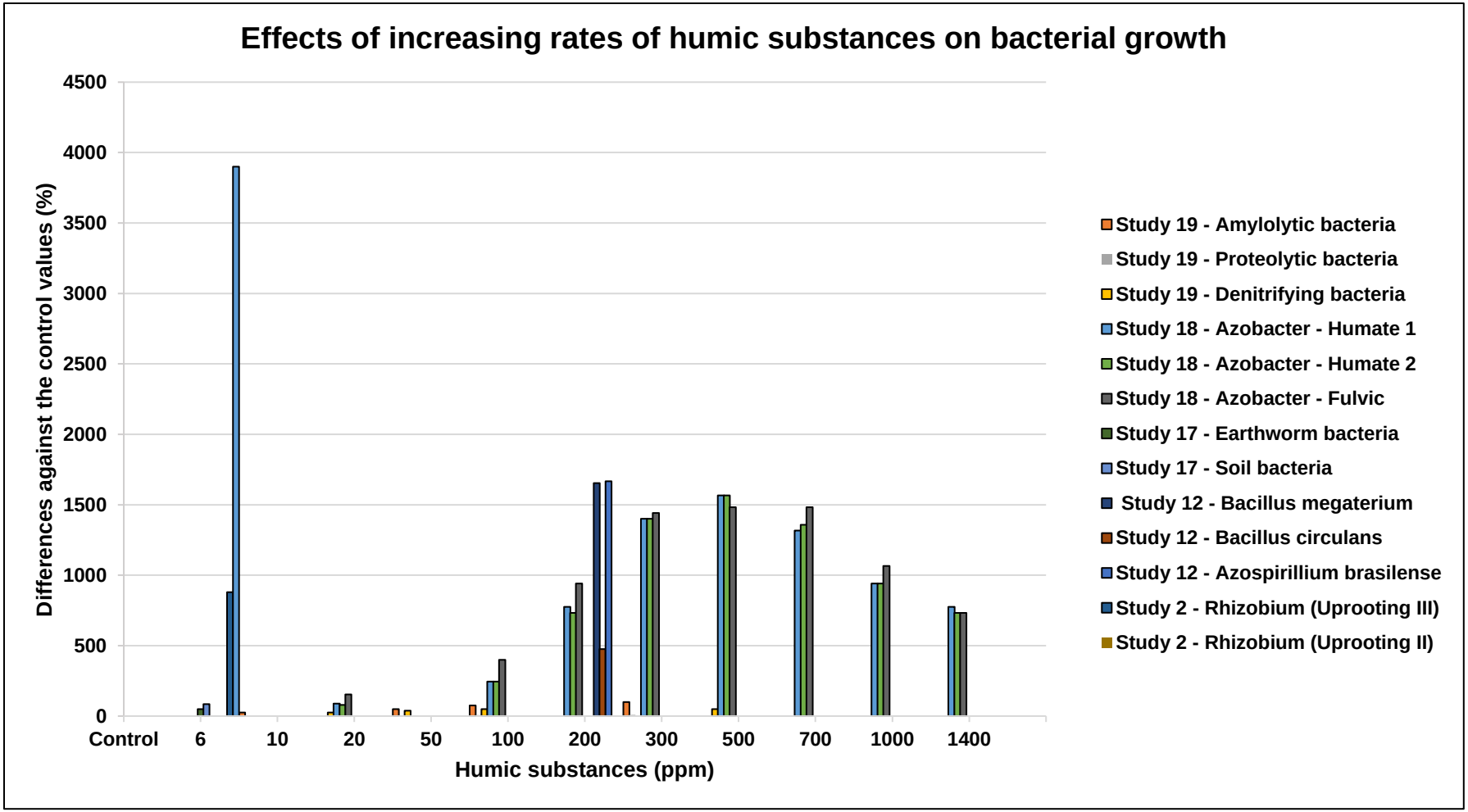

Figure 3. Responses of bacterial growth under increasing rates of humic substances. The parameters used to measure the bacterial growth were number of nodules (Study 2), most probable number (Study 12), maximal specific growth rate (Study 17), number of cells (Study 18) and number of microbes (Study 19).

CONCLUSION

This review has shown that the response of plant development and microbial activity to humic substances, although generally positive, is influenced by a number of environmental and management factors such as soil pH and application rate. These findings indicate that the source and application rate of humic substances and biofertilizers will have a strong impact on whether or not plant growth and microbial activity will significantly improve. The plant and microbial species also influence the response to humic substances. Furthermore, the interactions between each of these different factors in the presence of humic substances and biofertilizers can increase the variability of results. Therefore, it is complex to obtain predictable responses. These vast number of variabilities increase the need for experiments to characterize the synergetic relations of humic substances + plants + microbes naturally occurring in the soil + biofertilizers. Based on the number of studies presenting positive effects, we conclude by reiterating that the prospects for the conjunctive use of humic substances and biofertilizers to stimulate plant development and microbial activity in agricultural systems are theoretically substantial. However, there is a scarce number of publications addressing this topic and more research is necessary.

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COMPETING INTERESTS

No potential conflict of interest was reported by the authors.

AUTHORS' CONTRIBUTIONS

All authors read and approved the final manuscript.

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Chapter 2: Corn Response to an Integrated Plant Nutrition System (IPNS) with Humic Acid and Biofertilizers

ABSTRACT

Sustainable agriculture production depends on the development of methods that optimize nutrient cycling, minimize use of external inputs, and maximize input use efficiency according to the conditions of each region. The principle of an integrated plant nutrition system (IPNS) is to tailor plant nutrition and soil fertility management, taking advantage of the combined and harmonious use of inorganic, organic and biological resources. This greenhouse study investigated the individual and combined use of inorganic, organic and biological fertilizer resources for corn (*Zea mays* L.). We evaluated the effects of commercial synthetic fertilizers, humic acid products, compost/manure teas and bioinoculant as inorganic, organic and biological resources, respectively, and their synergy on corn growth and soil respiration parameters under a period of water stress. The pots were laid out in completely randomized design and the total of sixteen treatment combinations were replicated four times. In general, when comparing to the control values, the use of humic acid (HA), biofertilizers and the integration of both compounds generated significantly greater early season plant height, chlorophyll content, photosynthetic efficiency and shoot/root dry biomass. The soil substrate induced respiration was affected by only one biofertilizer product at two different rates. Though all pots received adequate fertilizer, the control plants were generally smaller and less vigorous compared to the plants receiving either HA or biofertilizer treatments, but no additive benefit was observed for the integrated practice compared to individual applications. Further studies addressing different types and levels of stress along with greater stress duration should be conducted to validate these findings.

Keywords: bioinoculants, compost tea, manure tea, organic fertilizer, humates, water stress, plant biostimulants

INTRODUCTION

Successful crop production relies on nutrients that are available in sufficient quantities and forms to promote satisfactory plant growth. Fertilization is an essential practice to enhance soil fertility, increase crop productivity and support agricultural intensification (Vaneeckhaute et al., 2013). Optimized fertilization schemes require methods to optimize nutrient cycling, minimize use of external inputs, and maximize input use efficiency appropriate to the conditions of each region (Cakmak, 2002; Kumwenda, Waddington, Snapp, Jones, & Blackie, 1996).

A huge variety of materials can serve as sources of plant nutrients. These can be inorganic, organic, recycled wastes or a range of biological products including compost teas and microbial inoculants. The nature and the characteristics of nutrient release from fertilizers derived from inorganic, organic and biological resources differ and thus must be managed differently (Chen, 2006; Dutta, Pal, Chakraborty, & Chakrabarti, 2003). Sustaining high crop yields should include not only the addition of synthetic fertilizer materials but also the integrated use of biological and organic nutrient resources as a way to increase nutrient use and minimize environmental impacts (Hussain, Jilani, & Iqbal, 1988; Kaur, Kapoor, & Gupta, 2005). According to the Food and Agriculture Organization of the United Nations (FAO) (Shand, 2007), the definition of an IPNS is “the adaptation of the plant nutrition and soil fertility management in farming systems to site characteristics, taking advantage of the combined and harmonious use of inorganic, organic and biological nutrient resources to serve the concurrent

needs of food production and economic, environmental and social viability." The principle of IPNS requires an understanding of nutrient dynamics throughout the soil-microbe-plant systems in order to regulate the availability of nutrients derived from inorganic, organic and biological sources to address long- and short-term crop production and environmental impacts (Aulakh & Grant, 2008).

Several studies addressing different crop species have shown the beneficial effects of the integrated use of different fertilizer or biostimulant sources on yield, shoot and root growth, and nutrient uptake on different species (Adesemoye, Torbert, & Kloepper, 2008; Mantelin & Touraine, 2004) such as sugarcane (*Saccharum officinarum* L.) (Bokhtiar & Sakurai, 2005; Sundara, Natarajan, & Hari, 2002) and red pepper (*Capsicum annuum* L.) (Joo, Kim, Lee, Song, & Rhee, 2004). However, in managed ecosystems, the dynamics of nutrient availability will vary depending on the nutrient resource applied.

The heterogeneous and complex molecules present in humic substances have shown many positive effects on plant growth, nutrient uptake efficiency and soil. Commonly, these effects can be intercorrelated and complementary. Plant growth stimulation from the use of HA has been reported in such ways as increased berry size and improved fruit quality in table grapes [*Vitis Vinifera* (L.) cv. Italia] (Ferrara & Brunetti, 2010) and greater root growth on Brazilian red cloak (*Megaskepasma erythrochlamys*) and sanchezia (*Sanchezia nobilis* L.) (Baldotto & Baldotto, 2014) and tobacco (*Nicotiana tabacum* L.) (Mylonas & McCants, 1980). Humic compounds stimulate plant development through improving nutrient adsorption on lily (*Lilium* L.) (Chang, Wu, Xu, Nikbakht, & Xia, 2012) and gerbera (*Gerbera jamesonii* L.) (Nikbakht et

al., 2008). Furthermore, humic acids can promote vegetal growth by mediating biochemical, morphological, physiological processes (Chen, Senesi, & Schnitzer, 1977; Tahiri, Destain, Thonart, & Druart, 2015; Vaughan & Malcolm, 1985).

The application of HA can positively affect soil cation exchange capacity and nutrient availability which indicates that HA materials may serve as resources that can improve fertilization efficiency (Albiach, Canet, Pomares, & Ingelmo, 2001; Tahiri et al., 2015; Vaughan & MacDonald, 1976). Thus, the physicochemical activity and structure of HA substances might increase agriculture production through improved soil quality and by enhancing soil stability and resistance to erosion (Brannon & Sommers, 1985; Spaccini, Piccolo, Conte, Haberhauer, & Gerzabek, 2002). The dual beneficial effects of humic acid on soil and plant might explain the production increase on tomato (*Solanum lycopersicum* L.), cotton (*Gossypium arboretum* L.) and grapes (*Vitis vinifera* L.) (Brownell, Nordstrom, Marihart, & Jorgensen, 1987) as improved soil promotes better conditions for plant growth.

Several studies have documented enhancement of vegetative growth, yield and nutrient uptake by improving the physico-chemical properties of the soil (Kim et al., 2015; Siddiqui, Islam, Naidu, & Meon, 2011) and incremental benefits to the microbial population for plants and soil fertility (Chen, 2015) in response to compost tea (CT) application. Moreover, solubilization of P or K, uptake of N and multiplication of extraradical hyphae biomass are effects promoted by biofertilizers that might minimize negative impacts of soil degradation, in addition to induction of plant growth (Bianciotto & Bonfante, 2002; Rodríguez & Fraga, 1999). Therefore, the application of microbial inoculants has shown the potential to improve sustainable production in

intensive agriculture systems (Bhattacharyya & Jha, 2012; Chauhan, Bagyaraj, Selvakumar, & Sundaram, 2015) due to nutrient release, plant growth stimulation, rhizoremediation and plant stress control (Lugtenberg & Kamilova, 2009).

In fact, the potential benefits of HA and biofertilizers as plant-growth promoters for increased nutrient acquisition (Yildirim, 2007), increased stress tolerance (Zhang & Ervin, 2004), and pathogen suppression (On et al., 2015) are evident in the literature, and substantial work has been done in this area. Thus, plant–microbial symbioses are very important components of nutrient cycling in agroecosystems and enhance plant nutrient uptake (Peoples & Craswell, 1992; Zhu, Cavagnaro, Smith, & Dickson, 2001). Studies have shown that HA stimulates microbial effects on ion exchange and metal complexing (chelating) systems (Puglisi et al., 2009; Visser, 1985). Also, HA has increased the production of micelium by mycorrhizal fungi (Gryndler et al., 2005) and promoted greater nodule formation of native rhizobia (Gaur & Bhardwaj, 1971).

Despite the recognition of the independent effects of HA and biofertilizers, few applicable studies have been conducted to elucidate the interaction of HA and biofertilizers on agronomic, economic and/or environmental outcomes. Moreover, there is a lack of knowledge regarding the effects of HA on plant–microbial symbioses. Therefore, the present study evaluated the effects of the combined and individual use of HA and compost/manure teas and bioinoculants along with inorganic fertilizer on corn (*Zea mays L.*) development and soil respiration. We hypothesize that the synergetic effects of the combination of HA + biofertilizer

will improve corn agronomic outcomes and increase soil respiration when comparing the application of each product alone.

MATERIALS AND METHODS

In this greenhouse study, humic acid (HA) was used as an organic resource and compost/manure tea and bioinoculants were used as biological resources along with conventional inorganic fertilizer resources (NPK) in an integrated manner.

2.1 Products Description (Treatments)

Seven products, including inorganic, organic and biological resources, were used in this study. The inorganic fertilizer was Osmocote Plus®; the organic product was MicroLife Humic Acid Complex®; and the biological products were SoilSoup®, Microgeo® and Microgro Supreme Bioinoculant®. The MicroLife 6-2-4® and Nanobind® are derived from organic and biological resources. The Osmocote Plus® is a slow release synthetic fertilizer containing 11 essential nutrients for plants. The organic/ humic category was represented by MicroLife Humic Acid Complex® which was constituted of 15% humic acid and 1% fulvic acid. One of the three biological fertilizers was Microgeo®, which is a Brazilian patented product categorized as a manure tea. This biofertilizer is composed of organic compounds, active and dormant cells from various microorganisms (bacteria, yeasts, filamentous fungi, and algae), metabolites and organo-mineral chelates and it is produced through continuous anaerobic fermentation in a liquid media (D'Andrea, 2002). According to the technical manual, the preparation is using the CLC® (Continuous Liquid Composting) process, where 5% of the commercial biological fertilizer Microgeo ®, 15% of ruminal content and water are mixed in a tank exposed to sunlight. After 15 days the biofertilizer is ready to be applied. SoilSoup® is an aerobic compost tea generated via

fermentation of vermicompost over 24 hours with the addition of nutrient solution (molasses, bat guano, sea bird guano, soluble kelp, langbeinite, natural citric acid, ancient seabed minerals, yucca) and oxygen to the system (aquarium pump). The Microgro Supreme Bioinoculant® is a water-soluble powder containing 76 different strains of bacteria and fungi including 11 different Mycorrhizal species and microbial food (sugars, humic acid, kelp, amino acids and yeast extract). The MicroLife 6-2-4® is a pelletized fertilizer that contains 6, 2 and 4% N, P and K, respectively. These nutrients are derived from a combination of organic and biological materials including fish, kelp, molasses, humates, bat guano, rock phosphate, wheat middlings, soy meal, cottonseed meal, alfalfa, corn meal, potassium sulfate, iron sulfate, Folic Acid, vitamins and bioinoculants. Nanobind® is constituted by the combination of humic substances and microbial inoculants. The products' descriptions are summarized in Table 1.

Table 1. Product description

Resource	Category	Subcategory	Name	Components
Inorganic	Synthetic		Osmocote Plus	Polymer-coated: Ammonium Nitrate, Ammonium Phosphate, Potassium Sulfate, Magnesium Sulfate, Sodium Borate, Iron Phosphate, Iron EOTA, Manganese Sulfate, Sodium Molybdate, Aibc Sulfate, Copper Sulfate and Zinc Oxide.
Organic	Humic	Fulvic	Microlife Humic Acid Complex	15% Humic Acid and 1% Fulvic Acid derived from leonardite
Organic + Biological	Biofertilizer	Manure tea	Microgeo	Recancitran Substances, Biodynamic Preparations, Pentoses, Minerals and Brans and the microorganisms produced in the manure tea fermentation
		Compost tea	SoilSoup	Molasses, Bat Guano, Sea Bird Guano, Soluble Kelp, Langbeinite, Natural Citric Acid, Ancient Seabed Minerals, Yucca and the microorganisms produced in the compost tea fermentation
		Bioinoculant	Microgro Supreme Bioinoculant	76 different strains of bacterias and fungi planced on dry milk carrier loaded with microbial food. The microorganisms included are: species of Genus Bacillus, Psuedomonas,Streptomycetes, Trichoderma, and Endo and Ectomycorrhizal Fungi
			Microlife 6-2-4	Fish, Kelp, Molasses, Emery Humates, Bat Guano, Rock Phosphate,Wheat Middling's, Soy Meal, Cottonseed Meal,Alfalfa, Corn Meal, Potassium Sulfate, Iron Sulfate, Amino Acids, Folic Acid,Vitamins and MicroGro Supreme BioInoculant
			Nanobind	Lactobacillus culture, Saccharomyces Boulardii culture, Phytase enzymes, Lipase enzymes, Amylase enzymes, Superoxide Dismutase enzymes, Protease enzymes, organic carbon (humic)

2.2 Experimental Design and Management

The experiment was conducted under controlled conditions in a greenhouse in Blacksburg (Virginia, USA) to investigate the individual and combined effects of humic acid (HA), compost/manure tea and bioinoculants on corn growth. Polyethylene pots (19 cm tall, 19 cm outside diameter, and 37851 cm^3 volume) were lined with plastic bags to avoid water loss. Soil media and sand (50% Metro-mix 360 and 50% playground sand, respectively) were placed in a polyethylene pot and 21 g of inorganic fertilizer (Osmocote Plus®) was equally added in each pot. According to the bulk density provided in the physical/chemical characteristics data sheet of each component, we added 0.425 kg Metro-mix 360 and 3 kg sand to each pot to have an equal volume. Posteriorly, corn seeds were planted by hand at 3 cm depth and thinned to one seedling after germination.

The field capacity on the soil media + sand was determined after water saturation until the first drop of water leached through the bottom of the pot. Then, after 1 day the weight of the pot containing the wet soil was taken to be used as field capacity threshold (Kirkham, 2014). We employed 6 treatments, each at two concentration levels, 1x and 2x the label rate of each product, depending on the treatment (Table 2). The trial used a completely randomized design (CRD) with four replications. Each treatment was applied at corn growth stages V1, V4, V6 and V8. The treatments were previously prepared in the laboratory and applied into each pot using an electronic pipette. Solid materials were dissolved in water and the appropriate rate applied to respective pots.

Table 2. Treatments and application rate

No.	Treatments			
	Product Name and Abbreviation	Rate	Label	Rate/pot (each application)
1.	Microgeo (M)	1x	150 l/ha	0.47 ml
2.	Microgeo (M)	2x	150 l/ha	0.94 ml
3.	Soil Soup (S)	1x	235 l/ha	0.73 ml
4.	Soil Soup (S)	2x	235 l/ha	1.46 ml
5.	Microgro Supreme Bio inoculant (MB)	1x	6.1 kg/ha	19 mg
6.	Microgro Supreme Bio inoculant (MB)	2x	6.1 kg/ha	38 mg
7.	Microlife Humic (H)	1x	14 l/ha	0.043 ml
8.	Microlife Humic (H)	2x	14 l/ha	0.086 ml
9.	Nanobind (N)	1x	4.6 l/ha	0.015 ml
10.	Nanobind (N)	2x	4.6 l/ha	0.030 ml
11.	Microlife 6-2-4(ML)	1x	975 kg/ha	3000 mg
12.	Microlife 6-2-4(ML)	2x	975 kg/ha	6000 mg
13.	Microgeo + Microlife Humic (M + H)	1x, 1x	150 l/ha and 14 l/ha	0.47 ml + 0.043 ml
14.	Soil Soup + Microlife Humic (S + H)	1x, 1x	235 l/ha and 14 l/ha	0.73 ml + 0.043 ml
15.	Microgro Supreme Bio inoculant + Microlife Humic (MB + H)	1x, 1x	6.1 kg/ha and 14 l/ha	19 mg + 0.043 ml
16.	Control (C)	0x	0	0

Note. The surface area on top of the pot was 314 cm^2

2.3 Water Regime and Data Collection

The pots were maintained at 60% of field capacity for the first 40 days of the experiment to ensure adequate moisture for corn growth. Between 40 and 50 days post-emergence (PE), watering was reduced to 30% of field capacity to induce mild to moderate drought stress. Plant height at the leaves within the whorl, atLEAF chlorophyll meter value (FT Green LLC, Wilmington, DE) and photosynthetic efficiency/OS-50II fluorometer (Opti-Sciences, Tyngsboro, MA) measurements were collected from the latest fully developed leaf defined using the leaf collar method (Abendroth, W. Elmore, J. Boyer, & K. Marlay, 2011) at 20, 40 and 60 days PE. At 60 days post-emergence, the aboveground plant material was clipped at the soil surface and dried at 70˚C until a constant weight was achieved so that plant dry matter yield could be calculated. Corn growth stages corresponding to 20, 40 and 60 days post-emergence were V4, V6 and V8, respectively. After aboveground biomass harvest, roots were separated from the soil media + sand by shaking and root dry matter calculated in a similar manner to the shoot.

2.4 Substrate-induced respiration

A subsample of soil from the whole pot (300 g) was collected at the end of the growth period following aboveground and root biomass collection. Substrate-induced respiration (SIR) was performed to determine active microbial biomass in soil samples (Fierer, Schimel, & Holden, 2003). The collected samples were weighed (4g dry weight equivalent) into modified 250 ml centrifuge tubes modified with holes drilled in the tube caps and filled with rubber caulk to facilitate gas extraction. Soils were conditioned to an incubation temperature of 20˚C prior to the addition of substrate. To each sample, 8 ml of yeast substrate was added (12 g BD Bacto™ yeast extract/liter H_2O) and the sample was placed on a shaker for 1 hour. After thoroughly

mixing substrate and soil, the tubes were tightly sealed and flushed with CO_2 free air for 3 minutes. After incubation at 20°C for 5 hours, a syringe was used to remove 5 ml of headspace gas from the sealed tubes. Analysis of the sample was performed with a Licor model LI-7000 infrared gas analysis (IRGA) (LI-COR Corporate, Lincoln, NE) to determine CO_2 concentration and soil respiration rate (ug CO2/g dry soil/hour).

2.5 Data Analysis

Analysis of variance using PROC GLM of SAS 9.4 (SAS Institute, 2011) was conducted to evaluate treatment effects on plant height, atLEAF chlorophyll meter values, photosynthetic efficiency and root, shoot and total biomass. Differences between treatments and control means were separated using Dunnett's test and the t-test of the means were deemed significant differences when F-test values were $\alpha<0.05$ for the plant parameters and $\alpha<0.1$ for the SIR. Single-degree of freedom contrasts were used to determine significant differences between rates of the same product.

RESULTS AND DISCUSSION

3.1 Plant Height

Generally, treatments positively impacted plant height to a greater degree as the study progressed from 20 to 60 days PE (Table 3).

Table 3. Analysis of variance of the effects of IPNS treatments on plant height, atLEAF and Fluoremeter at 20, 40 and 60 days post-emergence (PE)

Source	Plant height			atLEAF			Fluorometer		
	20 days PE	40 days PE	60 days PE	20 days PE	40 days PE	60 days PE	20 days PE	40 days PE	60 days PE
					Pr > f				
Rep	0.2985	0.0047	<0.0001	0.1869	0.1316	0.0095	<0.0001	0.0113	0.5537
Treatment	0.044	0.0002	<0.0001	0.0089	0.1715	0.0265	<0.0001	<0.0001	<0.0001
CV	13.8	7.0	8.2	7.9	7.0	3.1	4.8	3.3	2.4
SED	6.1	4.2	10.3	4.5	3.8	2.0	0.034	0.026	0.019

Progressively, the number of treatments with plant height significantly greater than the control increased as the study progressed. The number of treatments with plants significantly taller than the control increased from 5 to 7 and then 11 at 20, 40 and 60 days PE, respectively (Table 4). Mbagwu and Piccolo (1997) tested the responses of coal-derived humic substances on corn and they also found increases in plant height, even though the plants in their research were generally shorter than this current study when comparing the plant height around 5 weeks after emergence. Several studies have shown the influence of biofertilizers on plant height stimulation of species like potato (*Solanum tuberosum* L.), tomato, maize (Bhattacharyya & Jha, 2012), tobacco (Zhang & Kong, 2014) and wheat (*Triticum aestivum* L.) (Aftab & Asghari, 2008). Moreover, a study on compost tea reported a pattern of increased plant height of lettuce (*Lactuca sativa* L.), soybean (*Glycine max* L.) and sweet corn as concentration of compost tea increased from 0.1%, 0.2%, 0.4%, to 0.8% of the total application (Kim et al., 2015).

Table 4. Mean height of control plants and differences between height of treatment and control at 20, 40 and 60 days PE.

Category	Treatment comparison	Difference between treatments and control values					
		20 days PE		40 days PE		60 days PE	
		Plant heigh, cm					
Biofertilizer	M - C (1x)	13.0	*	11.8	*	28.0	*
	M - C (2x)	14.9	*	11.8	*	31.5	*
	S - C (1x)	9.1		10.0	*	32.0	*
	S - C (2x)	12.1		14.8	*	37.2	*
	MB - C (1x)	11.1		14.3	*	32.6	*
	MB - C (2x)	10.8		10.8	*	36.2	*
Humic	H - C (1x)	8.9		7.0		30.6	*
	H - C (2x)	13	*	8.3		21.0	
Humic + Biofertilizer	N - C (1x)	11.4		4.0		3.4	
	N - C (2x)	4.8		4.5		16.2	
	ML - C (1x)	10.8		9.0	*	25.8	*
	ML - C (2x)	5.4		1.3		21.6	*
	M+H - C (1x,1x)	11.7		6.8		21.3	
	S+H - C (1x,1x)	15.9	*	8.8		22.9	*
	MB+H - C (1x,1x)	15.6	*	8.5		21.9	*
Actual Control values (C)		33.3		52.3		102.5	

Note. * denotes significant differences, $\alpha<0.05$

M = Microgeo; S = SoilSoup; MB = Microgro Supreme Bio Inoculant; H = Microlife Humic; N = Nanobind; ML = Microlife 6-2-4; C = Control.

Figure 1: Plant height visual contrast between control (left) and Microgeo 2x treated plants (right) at 60 days post-emergence.

3.2 Chlorophyll content (atLAEF) and Photosynthetic Efficiency (F_v/F_m)

The total photosynthetic pigments or chlorophyll content has been used to assess the physiological status of plants and to detect stress conditions such as high salt level in soil (Taïbi et al., 2016) and drought (Zhang et al., 2011). The statistical significance of atLEAF values were less drastic than that of fluorometer (Table 3). Generally, atLEAF values did not differ between treatments and the control with some values lower than the control (Table 5). At 40 days PE, water content in the pots was dropped to 30% of field capacity and the treatments combining HA + biofertilizer had greater atLEAF values at 60 days PE in response (Table 5). This may indicate that corn plants receiving these treatments suffered less stress from water limitation. In contrast to our results where differences in indirect measures of chlorophyll content were scarce, other

studies have reported that HA consistently increases chlorophyll content in potato leaf tissue (Selim, Shedeed, Asaad, & El-Neklawy, 2012) and roselle (*Hibiscus sabdariffa* L.) (Sanjari, Sirousmehr, & Fakheri, 2015) under hydric stress conditions. Azab (2016) revealed that biofertilizers alone and in combination with NPK increased chlorophyll content of corn under moderate, intermediate and severe water deficit. Also, the same study showed that biofertilizer + 50% NPK produced greater chlorophyll content than the application of biofertilizer + 100% NPK under normal irrigation and water deficit. Abdelraouf, El-Habbasha, Hozayn, and Hoballah (2013) found that the application of biofertilizer to wheat significantly increased total chlorophyll under 100%, 80%, 60% and 40% irrigation requirements compared to treatments without biofertilizer. Furthermore, to clarify the relationship between atLEAF and the actual chlorophyll content, devices that provide a non-destructive estimate of the amount of chlorophyll present in the plant leaf (Gianquinto et al., 2004) and strong relationships between these chlorophyll meters readings and the actual chlorophyll content in the leaves in many different crops (Pellizzaro, Ventura, Arca, & Canu, 1998) including corn (Castelli, Contillo, & Miceli, 1996; Markwell, Osterman, & Mitchell, 1995) have been reported. The SPAD-502 Chlorophyll meter (Soil Plant Analysis Development, Minolta Camera Co., Ltd., Japan) is the most used device, however the atLEAF Chl meter (FT Green LLC, Wilmington, DE) used in this study can be an affordable alternative to the SPAD-502 meter (Zhu, Tremblay, & Liang, 2012).

Photosynthetic efficiency values were higher than control values for most treatments in all three data collection periods (Table 6). A more energetic photosynthesis process could affect plant development such as greater plant height and biomass values measured in this study. According to Björkman and Demmig (1987) the optimal value of F_v/F_m is around 0.83 for most

species, depending on the developmental stage of the leaves, with lower values indicating plant stress. Thus, the photosynthetic efficiency readings collected in the most mature period (60 days PE) showed that the treatments presenting significant differences between control were much closer to the optimal/non-stress threshold. Lotfi et al. (2018) tested the effects of HA on photosynthetic efficiency of rapeseed (*Brassica napus* subsp. *napus*) plants in different water regimes and the application of HA resulted in increased maximum quantum yield of PSII photochemistry (F_v/F_m), where the highest discrepancy between non-humic acid and HA treatments appeared in response to the most severe water stress. Shool and Shamshiri (2014) tested the interaction effect of mycorrhizal fungi *Glomus mosseae* and the bacterial strain *Pseudomonas fluorescens* P_{52} in pistachio (*Pistacia vera*) cv. Qazvini plants under water regimes of 100%, 75%, 50% and 25% of field capacity and the highest discrepancy of F_v/F_m values between non-biological and biological fertilization appeared in the treatments managed under 25% of field capacity.

The chlorophyll works as a photoreceptor in photosynthesis, thus there are studies showing the correlation between total chlorophyll content and F_v/F_m in aloe vera (*Aloe vera*) (Hazrati, Tahmasebi-Sarvestani, Modarres-Sanavy, Mokhtassi-Bidgoli, & Nicola, 2016), olive tree (*Olea europaea*) (Khaleghi, Arzani, Moallemi, & Barzegar, 2012) and wheat (Sharma, Andersen, Ottosen, & Rosenqvist, 2015). These studies showed lower values of total chlorophyll and F_v/F_m during water or heat stress and higher values when the plants were experiencing ideal conditions. When comparing these previous studies with this current study, we found similar relationships for chlorophyll content and F_v/F_m. However, the chlorophyll content and F_v/F_m relationships in our study were not as evident as the values presented in the three studies

mentioned before. In fact, the difference between control and treatments was more evident in the F_v/F_m than atLEAF / chlorophyll content readings (Table 5 and 6).

Table 5. atLEAF readings represented by the actual control values and the difference between treatment and control values

Category	Treatment comparison	Difference between treatments and control values					
		20 days PE		40 days PE		60 days PE	
		atLEAF, unit					
Biofertilizer	M - C (1x)	3.63		7.73		2.08	
	M - C (2x)	2.95		5.55		1.93	
	S - C (1x)	5.10		7.20		3.53	
	S - C (2x)	4.15		6.70		1.33	
	MB - C (1x)	3.85		7.92	*	3.03	
	MB - C (2x)	5.08		5.33		3.93	
Humic	H - C (1x)	7.28		5.30		4.15	
	H - C (2x)	-0.03		6.88		3.93	
Humic + Biofertilizer	N - C (1x)	-2.48		3.15		2.83	
	N - C (2x)	9.08		4.45		4.03	
	ML - C (1x)	8.03		5.93		4.98	*
	ML - C (2x)	8.80		5.20		1.48	
	M+H - C (1x,1x)	6.78		7.48		4.80	*
	S+H - C (1x,1x)	10.15	*	10.02	*	4.87	*
	MB+H - C (1x,1x)	6.20		6.98		4.30	*
Actual Control Values (C)		52.23		47.85		60.88	

Note. * denotes significant differences, $\alpha<0.05$
M = Microgeo; S = SoilSoup; MB = Microgro Supreme Bio Inoculant; H = Microlife Humic; N = Nanobind; ML = Microlife 6-2-4; C = Control.

Table 6. OS-50II fluorometer readings (Photosynthetic efficiency) represented by the actual control values and the difference between treatment and control values

Category	Treatment comparison	Difference between treatments and control values					
		20 days PE		40 days PE		60 days PE	
		Fluorometer, Fv/Fm					
Biofertilizer	M - C (1x)	0.258	*	0.112	*	0.057	*
	M - C (2x)	0.260	*	0.123	*	0.070	*
	S - C (1x)	0.125	*	0.048		0.008	
	S - C (2x)	0.136	*	0.049		0.003	
	MB - C (1x)	0.248	*	0.117	*	0.074	*
	MB - C (2x)	0.272	*	0.129	*	0.069	*
Humic	H - C (1x)	0.238	*	0.131	*	0.031	
	H - C (2x)	0.211	*	0.106	*	0.031	
Humic + Biofertilizer	N - C (1x)	0.134	*	0.101	*	0.013	
	N - C (2x)	0.159	*	0.069	*	0.006	
	ML - C (1x)	0.248	*	0.114	*	0.070	*
	ML - C (2x)	0.246	*	0.133	*	0.049	*
	M+H - C (1x,1x)	0.311	*	0.136	*	0.071	*
	S+H - C (1x,1x)	0.225	*	0.113	*	0.046	*
	MB+H - C (1x,1x)	0.289	*	0.130	*	0.070	*
Actual Control Values (C)		0.489		0.669		0.742	

Note. * denotes significant differences, $\alpha<0.05$
M = Microgeo; S = SoilSoup; MB = Microgro Supreme Bio Inoculant; H = Microlife Humic; N = Nanobind; ML = Microlife 6-2-4; C = Control.

3.3 Plant biomass

Final shoot, root and total dry biomass were all significantly affected by IPNS treatment (Table 3). The greatest effect of IPNS treatment occurred for root biomass receiving HA treatments and shoot biomass receiving the biofertilizer treatments (Table 7). Total biomass was also affected by most of the treatments, where only 3 treatments did not present significantly higher value than control. Previous studies have reported increased shoot and root biomass when humic acid and/or biofertilizers were applied, especially under stress conditions (Dimkpa, Weinand, & Asch, 2009; Prado et al., 2016).

Shoot and root dry weight are commonly used to measure the effects of humic substances (Chen & Aviad, 1990) and in this corn study, the HA treatments resulted in the greatest root biomass. Chen and Aviad (1990) mentioned that the increased root growth promoted by HA is generally more evident than shoot growth, which is also what we observed in the current study. Other researchers have documented increased root biomass when HA was applied in soybeans (Prado et al., 2016), lettuce (Young & Chen, 1997), bentgrass (*Agrostis palutris*) (Dorer & Peacock, 1997), forage turnips (*Brassica rapa* L.) (Albayrak & Camas, 2005) and tomato (Adani, Genevini, Zaccheo, & Zocchi, 1998). In contrast, (Hartz & Bottoms, 2010) tested five commercial HA formulas and found no significant effect on tomato dry mass accumulation. Therefore, the effectiveness of humic acid will depend on product rate, severity of stress, organic matter content of the soil, HA composition and extraction method. According to Tahiri et al. (2015), humic substances influence two main mechanisms of plant growth: improvement of nutrient availability and phyto-stimulation. The use of HA enhanced the adsorption of macro and micro nutrients of gerbera (Nikbakht et al., 2008) and the presence of physiologically active concentrations of cytokinin in humic substances was demonstrated in a study using radish (*Raphanus sativus* L.) and corn plants (Pizzeghello, Francioso, Ertani, Muscolo, & Nardi, 2013). Though the effects of humic substances on root biomass have solid evidence, a number of studies also present beneficial effects of HA on length and fresh and dry weight of shoots (Nardi, Carletti, Pizzeghello, & Muscolo, 2009).

Biofertilizers most often affected shoot biomass (Table 7). Previous studies have reported significant increases in shoot dry biomass for wheat (Singh & Prasad, 2011), rice (*Oryza sativa* L.) (Yuwono, Handayani, & Soedarsono, 2005) and lettuce (Kohler, Caravaca, & Roldán, 2010)

when various biofertilizers were applied. Application of biofertilizers derived from vermicompost tea also outperformed the control in terms of shoot biomass on tomatoes (Edwards, Arancon, & Greytak, 2006; Fritz, Franke-Whittle, Haindl, Insam, & Braun, 2012). The plant growth effects caused by the use of biofertilizers have been attributed to increased microbial population, biologically active substances and nutrition promotion by accelerating mineralization processes (Rodríguez & Fraga, 1999; Somers, Vanderleyden, & Srinivasan, 2004). It was also postulated that the growth stimulation might be due to the phytohormones synthesizing as auxins (Dobbelaere, Croonenborghs, Thys, Broek, & Vanderleyden, 1999), gibberellic acids (Turan et al., 2014), and cytokinins (Zhang et al., 2014). Biofertilizer treatments alone affect root biomass to a much lesser extent comparing to the other materials (Table 7), however there are several studies showing the benefits of biofertilizers on root growth in several crops (Bhardwaj, Ansari, Sahoo, & Tuteja, 2014) and wheat (Dobbelaere et al., 1999).

In general, the use of HA and/or biofertilizers increased total plant biomass compared to the control, however the integrated use of these compounds interestingly resulted in plants with more proportional above/belowground biomass ratio. A lower shoot:root ratio (Table 7) could indicate greater stress tolerance at a more mature growth stage because a proportional root system may have improved ability to send nutrients/water to the aboveground biomass. In both greenhouse and field trials, Canellas et al. (2013) validated a synergistic effect of biofertilizer and HA, where corn grain yield was 45% and 48% higher with the integrated use of both compounds when comparing with the independent use of biofertilizer and HA, respectively.

Table 7. Shoot, root and total dry biomass readings represented by the actual control values and the difference between treatment and control values. Shoot:root ratio is an absolute value.

Category	Treatment comparison	Difference between treatments and control values						
		Shoot biomass		Root biomass		Total biomass		Shoot : root ratio
		g		g		g		g
Biofertilizer	M - C (1x)	25.25	*	4.65	*	29.90	*	3.77
	M - C (2x)	33.80	*	2.23		36.03	*	4.92
	S - C (1x)	34.75	*	1.68		36.43	*	5.17
	S - C (2x)	33.67	*	0.25		33.92	*	5.63
	MB - C (1x)	24.72	*	1.65		26.37	*	4.50
	MB - C (2x)	13.33		2.38		15.71		3.56
Humic	H - C (1x)	18.60		6.25	*	24.85	*	3.12
	H - C (2x)	11.78		7.02	*	18.80		2.66
Humic + Biofertilizer	N - C (1x)	14.13		6.60	*	20.73	*	2.84
	N - C (2x)	8.08		6.00	*	14.08		2.61
	ML - C (1x)	24.67	*	5.57	*	30.24	*	3.56
	ML - C (2x)	13.00		3.40		16.40		3.32
	M+H - C (1x,1x)	12.10		8.77	*	20.87	*	2.47
	S+H - C (1x,1x)	17.90		6.95	*	24.85	*	2.98
	MB+H - C (1x,1x)	12.28		12.27	*	24.55	*	2.13
Actual Control Values (C)		42.10		13.23		55.33		3.19

Note. * denotes significant differences, $\alpha<0.05$

M = Microgeo; S = SoilSoup; MB = Microgro Supreme Bio Inoculant; H = Microlife Humic; N = Nanobind; ML = Microlife 6-2-4; C = Control.

Figure 2: Root biomass visual contrast between control (left) and Microgro Supreme Bio Inoculant + Microlife Humic (right).

3.4 Differences between the two application rates of the same product.

The treatments using only one product were tested at 1x and 2x the label rate per application. The statistically significant differences between the two application rates (1x and 2x) were scarce considering the products tested and parameters collected. MicroLife 6-2-4 at the lower rate (1x) resulted in greater differences in total biomass, plant height (40 days PE) and atLeaf (60 days PE) (Table 8). Lower rates (1x) of Microlife humic produced greater atLEAF values at (20 days PE) (Table 8). The only product where doubling the rate generated significant greater difference was the application of Nanobind (2x) on atLEAF (20 days PE) (Table 8). Thus, it might be reasonable to affirm that the companies are providing a proper label rate for each product considering the two rates tested in this study. Nikbakht et al. (2008) have tested five different levels of humic acid and found that higher levels decreased absorption of some nutrients, confirming the importance of suitable application rate. Furthermore, Vallini, Pera, Avio, Valdrighi, and Giovannetti (1993) found the optimal dose of humic acid + *Glomus mosseae* and the treatments where humic acid concentration was above the optimal dose the

laurel (*Laurus nobilis* L.) shoot and root fresh weight decreased to values lower than the control in which no humic acid was applied.

Table 8. Plant height contrast between 1x and 2x of the label application rate

Product	Plant height			atLEAF			Shoot biomass	Root biomass	Total biomass
	20 days	40 days	60 days	20 days	40 days	60 days			
	Pr>f								
Microgeo 1x vs. 2x	0.659	1.000	0.631	0.833	0.420	0.917	0.194	0.070	0.376
SoilSoup 1x vs. 2x	0.490	0.120	0.482	0.767	0.852	0.129	0.869	0.281	0.717
MicroGro Bioinoculant 1x vs. 2x	0.941	0.249	0.629	0.702	0.336	0.531	0.086	0.582	0.126
MicroLife Humic 1x vs. 2x	0.305	0.678	0.196	0.026 *	0.559	0.875	0.298	0.556	0.382
Nanobind 1x vs. 2x	0.127	0.868	0.084	0.001 *	0.629	0.404	0.356	0.648	0.337
MicroLife 6-2-4 1x vs. 2x	0.215	0.013 *	0.568	0.809	0.787	0.018 *	0.079	0.103	0.049 *

Note. * denotes significant differences, $\alpha<0.05$

3.5 Substrate-induced respiration (SIR)

Substrate-induced respiration uses the physiological respiration reactions of the microorganisms from the soil to measure microbial activity (Anderson & Domsch, 1978). According to Swaina, Bastiraya, Jitendraa, and Haibrub (2014), the SIR method offers a reliable and easy assessment of the microbial biomass and other aspects of microbial growth in the soil. The use of this method to evaluate the treatments tested in this study showed statistically significant differences (Table 9). Though SIR was positive for 11 of 15 IPNS treatments, only two treatments were significantly higher than the control (Table 10) and responses in general did not follow the same trend as the plant parameters. The two rates of Microgro Supreme Bioinoculant were the only treatments with SIR values greater than the control. Khan et al. (2015) reported a study testing different bioinoculants and vermicompost in combination and alone, where all treatments had higher soil respiration values than the control which did not

receive bioinoculants and vermicompost. Moreover, the same study presented soil respiration increment varying from 29.4% to 53.6% over the control value, depending on the treatment.

Application of HA did not significantly affect the SIR results. Hartz and Bottoms (2010) tested the effects of HA on microbial respiration in two different soils containing high and low organic matter. In their study, the addition of HA enhanced microbial respiration only in the low organic matter soil. Therefore, the high organic matter content present in our soil media may have decreased any potential HA influence on microbial respiration.

Table 9. Analysis of variance of the IPNS treatments on substrate-induced respiration.

Source	SIR
	Pr > f
Rep	0.1012
Treatment	0.00
CV	50.0
SED	2.9

Table 10. Substrate-induced respiration (SIR) values represented by the actual control values and the difference between treatment and control values.

Category	Treatment comparison	Difference between treatments and control values Substrate-induced respiration (SIR) ug CO2 / g dry soil / hour	
Biofertilizer	M - C (1x)	-0.50	
	M - C (2x)	0.40	
	S - C (1x)	0.10	
	S - C (2x)	0.20	
	MB - C (1x)	9.10	*
	MB - C (2x)	5.80	*
Humic	H - C (1x)	-0.70	
	H - C (2x)	-0.20	
Humic + Biofertilizer	N - C (1x)	0.20	
	N - C (2x)	-0.20	
	ML - C (1x)	1.00	
	ML - C (2x)	1.60	
	M+H - C (1x,1x)	0.10	
	S+H - C (1x,1x)	0.90	
	MB+H - C (1x,1x)	2.90	
Actual Control Values (C)		4.50	

Note. * denotes significant differences, $\alpha<0.01$

CONCLUSIONS

The individual and combined use of HA and biofertilizers generally increase corn growth and development parameters under the conditions of this study. Though all pots received adequate fertilizer, the control plants were generally smaller and less vigorous compared to the plants receiving either HA or biofertilizer treatments, but no additive benefit was observed for the integrated practice compared to individual applications. At 40 days PE the biofertilizer products Microgeo, SoilSoup and Microgro Supreme Bioinoculant all produced plants that were taller than the control. This remained the case at 60 days PE when taller plants were also associated with application of MicroLife 6-2-4. In general, shoot dry matter was increased by the biofertilizer products Microgeo, SoilSoup and Microro Supreme Bioinoculant while root dry

matter was most positively affected by humic acid and Nanobind, a phenomenon commonly noted in previous research. Impacts on total biomass were mixed based on contributions of increased root biomass, shoot biomass or both with 11 of 15 treatments exhibiting greater total biomass than the control. Applying twice the labeled rate rarely resulted in observations that were different from the recommended rate, likely indicating that instructions for use of these products are currently appropriate when comparing the two rates tested in this study. Differences in atLeaf chlorophyll meter readings were uncommon for any treatment in our study. However, all treatments had higher fluorometer readings at 20 days PE and higher readings for 13 of 15 treatments at 40 days PE. Although the current study cannot affirm that the conjunctive use of HA and biofertilizers is a better practice than the application of each compound alone, we did find positive benefits from the application of these compounds to corn. Further studies addressing different types and levels of stress and greater stress duration should be conducted to validate these findings and contribute further understanding of the value of the IPNS approach.

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Chapter 3: Integrated Plant Nutrition System (IPNS) effects on Cannabis sativa development

ABSTRACT

The illegal status of cannabis (*Cannabis sativa* L.) post-World War II resulted in a lack of research on agricultural practices and insufficient information on water use, fertilizer, disease control and cropping systems. However, there is a resurgence of interest in cannabis due to diverse uses such as a rich source of cellulosic/woody fiber and construction uses, seed oil, bioenergy and pharmaceutical properties. Cannabis can be a suitable crop for alternative agricultural practices due to its resistance to many pathogens and herbivores. 'Cherry Kandy' is a strain used for cannabidiol (CBD) extraction and there is an expanding industry using cannabis extracts to minimize anxiety, epileptic seizures and pain. The principle of an integrated plant nutrition system (IPNS) is to enable the adaption of the plant nutrition and soil fertility management in farming systems to site characteristics, taking advantage of the combined and harmonious use of inorganic, organic and biological resources to serve the concurrent needs of food production, economic, environmental and social viability. This project investigated the individual and combined use of inorganic, organic and biological fertilizer resources on cannabis under a period of moderate water stress. We evaluated the effects of commercial synthetic fertilizer, humic acid compound (HA), manure tea and bioinoculant as inorganic, organic and biological resources, respectively and their synergy on cannabis development. Our hypothesis was that the synergetic effects of HA + biofertilizers would improve cannabis growth especially under water stress. The greenhouse study tested cannabis growth and stress parameters. The pots were laid out in completely randomized design and the total of nine treatment combinations were

replicated four times. The results of this study indicated that application of HA and biofertilizer alone, or in combination, generally increased plant height, chlorophyll content and photosynthetic efficiency, especially immediately after the water stress. Cannabis biomass of treated plants was rarely different from the control. Applying twice the labeled rate (2x) generally did not result in measurements that were different from the recommended rate (1x). Also, the combined application of HA + biofertilizer did not show a clear evidence of additional benefits. Future research should focus on the effects of biostimulants on CBD/THC content due to the potential impact on the production of secondary metabolites in plants under stress.

Keywords: cannabis, hemp, biostimulants, humic acid, biofertilizer, manure tea, water stress

INTRODUCTION

Cannabis is classified into the family Cannabaceae and has three main types: C. *sativa*, C. *indica*, and C. *ruderalis* (Stearn, 1970) and has various prospective uses in the industrial, food and medical sector (Fike, 2016). However, after the Second World War cannabis cultivation decreased significantly (Callaway, 2004) and research, environmental impacts and legal human experience with this plant lost its importance (Eisenstein, 2015). According to Butsic and Brenner (2016) this lack of research on cannabis agricultural practices caused by the illegal status of the plant resulted in a lack of information related to water use, fertilizer and disease control requirement, cropping systems and yields.

Generally, cannabis agriculture has been underemphasized in terms of its potential to compete with other crops and, therefore, to become a regular source of income (Weisheit, 2011).

However, hemp is receiving renewed interest due to its multi-purpose application as a rich source of cellulosic/woody fiber and construction uses (Andre, Hausman, & Guerriero, 2016), seed oil (Kriese et al., 2004), bioenergy (Finnan & Styles, 2013; Sausserde & Adamovics, 2013) and pharmaceutical properties (Zuardi, 2006). Regarding the pharmaceutical utilization, there is expanding industry using cannabis extracts, specially cannabidiol (CBD), to minimize anxiety (Hagerty, Williams, Mittal, & Hutchison, 2015), epileptic seizures (Detyniecki & Hirsch, 2015; Rosenberg, Tsien, Whalley, & Devinsky, 2015) and pain (Jensen, Chen, Furnish, & Wallace, 2015). The cultivar 'Cherry Kandy' is a strain with high CBD/THC ratio, mostly used for CBD extraction. Currently, there are a huge number of cannabinoids substances and the most abundant are cannabidiol (CBD), 9-tetrahydrocannabinol (THC), cannabigerol (CBG) and cannabichromene (CBC) (De Zeeuw, Malingre, & Merkus, 1972; Holley, Hadley, & Turner, 1975).

Some cannabis products are considered high value and the cultivation of this plant may be an alternative for small-scale farmers (Butsic & Brenner, 2016) or those looking to improve profit per area. According to Werf (1994), cannabis can be a suitable crop for alternative agricultural practices such as organic agriculture, due to natural resistance to many pathogens and herbivores. However, cannabis is known to be susceptible to common diseases of other crops, such as Tobacco Mosaic Virus (Shivprasad et al., 1999). The popular belief is that cannabis as a relative low input crop (Seleiman, Santanen, Kleemola, Stoddard, & Mäkelä, 2013) and adaptable to marginal soils, however several studies have shown that cannabis production requires adequate fertilizer supply to optimize yields (Adamovics, Ivanovs, & Stramkale, 2016; Iványi, 2011). Therefore, the cannabis growers need an effective fertilization strategy to reach

higher yields. According to the Food and Agriculture Organization of the United Nations (FAO) (Shand, 2007), the definition of an Integrated Plant Nutrition System (IPNS) is “the adaptation of the plant nutrition and soil fertility management in farming systems to site characteristics, taking advantage of the combined and harmonious use of inorganic, organic and biological nutrient resources to serve the concurrent needs of food production and economic, environmental and social viability”.

Essential plant nutrients such as N, P and K from synthetic fertilizers are commonly used by conventional farmers, however there are other potential fertilizer products originating from organic and biological sources. Humic acid (HA) compounds are categorized as an organic resource and there are many studies showing the effects of HA on plant development. El-Ghamry, El-Hai, and Ghoneem (2009) tested the application of different HA and amino acids on Faba Beans (*Vicia faba* L.) and found that the adequate rate significantly increased plant height, yield components, micronutrients adsorption and chlorophyll concentration. The proper HA rate also significantly increased root growth and nitrogen uptake of container-grown olive (*Olea europea* L.) plants (Tattini, Bertoni, Landi, & Traversi, 1991). Studies addressing biofertilizer products originating from biological resources such as compost / manure teas and bioinoculants, have shown various benefits for plants. Wang, Radovich, Pant, and Cheng (2014) found that chicken manure-based vermicompost tea suppressed the abundance of plant-parasitic nematodes on zucchini (*Cucurbita pepo* L.) plants. Vermicompost extract stimulated seed germination, hypocotyl and radicle growth as well as increased chlorophyll content in cannabis (Ievinsh, Vikmane, Ķirse, & Karlsons, 2017). Conant, Walsh, Walsh, Bell, and Wallenstein (2017) tested

a bioinoculant on cannabis and in addition to greater bud yield bioinoculant application also led to significant increases in plant height and basal steam area.

Plant biomass increase has been documented in response to the application of humic acid (Chen & Aviad, 1990) and biofertilizers (Bashan & de-Bashan, 2010; Pereg, de-Bashan, & Bashan, 2016). According to Turner, Hemphill, and Mahlberg (1978), cannabinoid compounds are present in all aerial part of the cannabis plant, mainly in bracts and leaves. Thus, the utilization of this IPNS method with humic acid and biofertilizers can increase cannabis biomass and it might be beneficial for higher cannabinoid concentration on plants or even increment of fiber yield (Sausserde & Adamovics, 2013) depending on the intention of each grower. Furthermore, in an online survey, Werse (2016) found that 43% of cannabis growers from Germany, Austria and Switzerland confirmed to have ecological ideology / activist reasons to grow cannabis. Therefore, cannabis growers might be willing to accept more environmentally friendly agricultural methods such as IPNS when compared to conventional farmers. Previous research demonstrating beneficial outcomes of the use of HA and biofertilizers on various plants and the inclination of cannabis growers to embrace more sustainable practices created the interest to conduct this study. Therefore, this study evaluated the effects of an IPNS with the combined and individual use of HA and manure tea and bioinoculants along with inorganic fertilizer on cannabis development including plant height, chlorophyll amount (atLEAF), photosynthetic efficiency and biomass under greenhouse condition.

MATERIALS AND METHODS

In this greenhouse study, humic acid (HA) was used as an organic resource and manure tea and bioinoculants were used as biological resources along with conventional inorganic fertilizer resources (NPK) in an IPNS.

2.1 Products description

Four products, including inorganic, organic and biological resources, were used in this study. The inorganic fertilizer was Osmocote Plus®; the organic product was MicroLife Humic Acid Complex®; and the biological products were Microgeo® and Microgro Supreme Bioinoculant®. Osmocote Plus® is a slow release synthetic fertilizer containing 11 essential nutrients for plants. The humic category was represented by MicroLife Humic Acid Complex® which was constituted mainly by 15% humic acid and 1% fulvic acid. One of the two biological fertilizers was Microgeo® which is a Brazilian patented product categorized as a manure tea. This biofertilizer is composed of organic compounds, active and dormant cells from various microorganisms (bacteria, yeasts, filamentous fungi, and algae), metabolites and organo-mineral chelates and it is produced through continuous anaerobic fermentation in a liquid media (D'andrea, 2002). According to the technical manual, the preparation uses the CLC® (Continuous Liquid Composting) process, where 5% of the commercial biological fertilizer Microgeo ®, 15% of ruminal content and 80% water are mixed in a tank at sunlight. After 15 days, the biofertilizer production is complete. The Microgro Supreme Bioinoculant® is a water-soluble powder containing 76 different strains of bacteria and fungi including 11 different Mycorrhizal species and microbial food (sugars, humic acid, kelp, amino acids and yeast extract). Products derived from humic acid and biological sources are broadly categorized as biostimulants. Products descriptions are summarized in Table 1.

Table 1. Product description and components

Resource	Category	Subcategory	Name	Components
Inorganic	Sinthetic		Osmocote Plus®	Polymer-coated: Ammonium Nitrate, Ammonium Phosphate, Potassium Sulfate, Magnesium Sulfate, Sodium Borate, Iron Phosphate, Iron EOTA, Manganese Sulfate, Sodium Molybdate, Aibc Sulfate, Copper Sulfate and Zinc Oxide.
Organic	Humic	Fulvic	Microlife Humic Acid Complex®	15% Humic Acid and 1% Fulvic Acid derived from leonardite
Organic + Biological	Biofertilizer	Manure tea	Microgeo®	Recancitrant Substances, Biodynamic Preparations, Pentoses, Minerals and Brans and the microorganisms produced in the manure tea fermentation
		Bioinoculant	Microgro Supreme Bioinoculant®	76 different strains of bacteria and fungi planced on dry milk carrier loaded with microbial food. The microorganisms included are: species of Genus Bacillus, Psuedomonas,Streptomycetes, Trichoderma, and Endo and Ectomycorrhizal Fungi

2.2 Experimental Design and Management

The experiment was conducted under controlled conditions in a greenhouse in Blacksburg Virginia, USA to investigate the individual and combined effects of humic acid (HA), manure tea and bioinoculant application on cannabis (*Cannabis sativa* L.) cultivar 'Cherry Kandy' growth including plant height, chlorophyll amount, photosynthetic efficiency and biomass under greenhouse condition. Polyethylene pots (19 cm tall, 19 cm outside diameter, 37,850 cm^3 volume and 314 cm^2 surface area) were lined with plastic bags to avoid water loss. Soil media and sand (50% Metro-mix 360 and 50% playground sand, respectively) were placed in a polyethylene pot and 22 g of inorganic fertilizer (Osmocote Plus®) was equally added to each pot, following the recommended application rate of 9.8 kg/100 m^2. According to the bulk

density provided in the physical/chemical characteristics data sheet of each component, we added 0.425 kg Metro-mix 360 and 3 kg sand to each pot to have an equal volume. Separately, the cannabis feminized seeds were planted in a seedling transplant tray filled with soil media (Pro-mix general purpose®) at 3 cm depth. Seedlings were transplanted to pots at 12 days post-emergence at which time the growth stage was three true leaf pairs on the BBCH scale (Mishchenko et al., 2017).

The field capacity of the soil media + sand was determined by saturating the media until water was observed dripping from the bottom of the pot. One day later, the weight of the wetted pot was used to estimate field capacity threshold (Kirkham, 2014).

There was a total of 9 treatments with each individual product applied at 1x and 2x the label rate (Table 2). The treatments combining biofertilizers + HA received only the label rate of each product (Table 2). The trial utilized a completely randomized design (CRD) with five replications. The application of each treatment occurred at cannabis growth stages 4, 6, 7 and 9 true leaf pairs (compound). The treatments were previously prepared in the laboratory and applied to each pot using an electronic pipette. Solid materials were dissolved in water and the appropriate rate applied to respective pots. According to the label of each product, the dilution rates were: Microgeo, 150 l/ha; Microgro Supreme, 6.1 kg/ha diluted in 233 liters of water and Microlife Humic, 14 l/ha diluted in 233 liters of water. The application of each treatment followed the dilution rate of the products and the proportions were properly adjusted according to the size of the pot.

Table 2. Treatments and application rates

No.	Treatments				
	Subcategory	Product Name and Abbreviation	Rate	Label	Rate/pot (each application)
1	Biofertilizer	Microgeo® (M)	1x	150 l/ha	0.47 ml
2.		Microgeo® (M)	2x	150 l/ha	0.94 ml
3.		Microgro Supreme Bioinoculant® (MB)	1x	6.1 kg/ha	19 mg
4.		Microgro Supreme Bioinoculant® (MB)	2x	6.1 kg/ha	38 mg
5.	Humic	Microlife Humic® (H)	1x	14 l/ha	0.043 ml
6.		Microlife Humic® (H)	2x	14 l/ha	0.086 ml
7.	Humic + Biofertilizer	Microgeo + Microlife Humic (M + H)	1x, 1x	150 l/ha and 14 l/ha	0.47 ml + 0.043 ml
8.		Microgro Supreme Bioinoculant + Microlife Humic (MB + H)	1x, 1x	6.1 kg/ha and 14 l/ha	19 mg + 0.043 ml
9.		Control (C)	0x	0	0

2.3 Water Regime and Data Collection

The pots were maintained at 60% field capacity during most of the experiment to ensure adequate moisture for cannabis growth. Between 35 and 42 days post-emergence (PE), watering was reduced to 30% of field capacity to induce mild to moderate drought stress. Plant height at the last shoot apex, atLEAF chlorophyll meter value (FT Green LLC, Wilmington, DE) and photosynthetic efficiency/OS-50II fluorometer (Opti-Sciences, Tyngsboro, MA) measurements were collected from the latest fully developed leaf at 33, 42 and 55 days PE. At 55 days PE, aboveground plant material was clipped at the soil surface and dried at 70°C until a constant weight was achieved, and plant dry matter yield calculated. Cannabis growth stages corresponding to 33, 42 and 55 days post-emergence were 7, 9 and 11 true leaf pairs (compound), respectively. Prior to sampling at 55 days PE (11 true leaf pairs) plants were still receiving applications. After aboveground biomass harvest, roots were separated from the soil media + sand by shaking and root dry matter calculated in a similar manner to the shoot.

2.4 Data analysis

Analysis of variance was conducted utilizing PROC GLM available in SAS 9.4 (SAS Institute, 2011) to evaluate treatment effects on plant height, atLEAF chlorophyll meter, photosynthetic efficiency, plant root and shoot, and total plant biomass. Before data analysis, an outlier test was performed utilizing a studentized residual and outliers identified when studentized residual was greater than 2.5. Mean separations were performed using the Tukey-Kramer command within the LSMEANS statement when F-tests indicated that significant differences existed ($p<0.05$).

RESULTS AND DISCUSSION

3.1 Plant Height

In general, the biostimulants which include the treatments with biofertilizers, HA and the combination of both, referring to all the treatments of this study except control, produced taller plants at all three timings. Plant heights collected at 33 days PE and after the water stress period (42 days PE) were more frequently greater than the control (Table 3) than at 55 days PE which had the fewest treatments that were different from the control (Table 3).

Table 3. Effects of biostimulant treatments on cannabis plant height at 33, 42 and 55 days post-emergence (PE).

Group	Category	Treatment	33 days PE		42 days PE		55 days PE	
			Plant height, cm					
None	None	Control	15.2	b†	22.86	b	37.6	b
Biostimulant	Biofertilizer	Microgeo (M) 1x	24.9	a	35.6	a	51.3	ab
		Microgeo (M) 2x	27.9	a	40.1	a	60.5	a
		Microgro Bio (MB) 1x	22.4	a	35.1	a	52.3	ab
		Microgro Bio (MB) 2x	21.8	ab	35.1	a	54.4	ab
	Humic	Microlife Humic (H) 1x	22.8	a	35.1	a	58.4	a
		Microlife Humic (H) 2x	22.4	a	33.5	ab	52.8	ab
	Humic + Biofertilizer	M + H	23.9	a	34.5	a	47.2	ab
		MB + H	23.4	a	34.0	ab	49.8	ab

† Means within a column followed by the same letter are not significantly different at the 0.05 probability level.

Cannabis sativa treated with a biofertilizer containing several beneficial bacteria culture grew significantly taller than control (Conant et al., 2017). Moreover, this biofertilizer called Mammouth P ™, led to a 16.5% increase in bud yields in Cannabis plants mostly likely due its effects on plant height and basal stem area. Pagnani et al. (2018) assessed the morphological and physiological effects of four fertilization strategies: nitrogen; inoculum 1; inoculum 2 and unfertilized control on cannabis. In this study, Pagnani et al. (2018) demonstrated that the biofertilization with plant growth-promoting rhizobacteria (PGPR) (inoculum 1) and nitrogen promoted significantly greater stem length than the control while Inoculum 2 was not different from the control. Moreover, the difference between inoculum 1 and 2 was the density of bacteria in the solution. Therefore, these findings do not necessarily agree with this current study in terms of biofertilizers with different rates or microorganism concentration may perform differently. In this sense, the two biofertilizer rates tested in this current study can theoretically lead to a different density of bacteria applied in the pot but we measured no effect on plant height (Table

3). Regarding the optimum biostimulant rate, previous research has reported increased plant height of lettuce (*Lactuca sativa* L.), soybean (*Glycine max* L.) and sweet corn with concentration of compost tea increasing from 0.1%,0.2%, 0.4%, to 0.8% of the total application (Kim et al., 2015). On the other hand, Dobbelaere, Croonenborghs, Thys, Vande Broek, and Vanderleyden (1999) presented a study where higher concentration of A. *brasilense* inoculum decreased wheat (*Triticum aestivum cv. Soissons*) growth and development compared to the control. We noted few effects of application rate on plant height. Studies have shown that the use of biofertilizers containing one or more beneficial microorganisms alleviate the imposed water stress in a greenhouse environment, increasing shoot and root growth of corn (Casanovas, Barassi, & Sueldo, 2002) and asparagus (*Asparagus officinalis* L.) (Liddycoat, Greenberg, & Wolyn, 2009). In our studies, the application of biofertilizers alone increased cannabis height compared to the control, mainly during the post water stress period measured at 42 days PE.

Caplan, Dixon, and Zheng (2017) tested increasing doses of an organic fertilizer containing HA on cannabis and reported that the middle-range dose (389 mg N/L) had higher growth index. Increasing the dose of the organic fertilizer consequently would increase the concentration of HA applied potentially explaining the greater growth index. According to du Jardin (2012), the effects of HA on plant growth can vary depending on its interactions with the environment, however the results are generally positive. Humic acid are known to minimize drought stress effects. However, our data collected immediately following the water stress period (42 days PE), showed that only half of the treatments that received HA had significantly taller plants. When comparing all data collection periods, the treatments containing HA (alone and in combination) at 42 days PE were more frequently different from the control than at 55 days PE

but less frequent than 33 days PE. Similar to what we observed with cannabis, application of HA increased growth of bluegrass (*Poa pratensis* L.) (Zhang & Schmidt, 1999) and rice (*Oryza sativa* L. cv. IACUB-30) (García et al., 2012) under water stress conditions.

The biofertilizer + HA treatments were not different from independent application of biofertilizers or HA at data collection periods.

Figure 1. Plant height visual contrast between Microgeo 2x treated plants (left) and control (right) at 55 days post-emergence.

3.2 Chlorophyll amount (atLEAF)

The physiological status and stress conditions of plants are often accessed through non-destructive measures such as estimating chlorophyll content (Jaleel et al., 2009) and photosynthetic efficiency (Ruiz-Sánchez, Aroca, Muñoz, Polón, & Ruiz-Lozano, 2010). The atLEAF value provides an indirect estimation of the actual chlorophyll content of the leaves. Gianquinto et al. (2004) assessed the reliability of hand-held chlorophyll meter in different

scenarios. They cited studies and affirmed that a strong correlation between chlorophyll meter readings and chlorophyll content of leaf laminae was found in more than 30 different species. In the same study, Gianquinto et al. (2004) stated that currently the SPAD-502 Chlorophyll meter (Soil Plant Analysis Development, Minolta Camera Co., Ltd., Japan) is the most used devise. However, Zhu, Tremblay, and Liang (2012) compared the SPAD and atLEAF Chl meter (FT Green LLC, Wilmington, DE) values of several crop species and reported strong correlation among laboratory-measured leaf chlorophyll content, SPAD values, and atLEAF values.

atLEAF values for humic and products were higher than the control at 33 and 42 days PE, while only the values for humic + biofertilizer were higher at 55 days PE (Table 4). While atLEAF values were inconsistently affected by treatment, only the two rates of Microlife Humic were not statistically greater than the control at 55 days PE. Previous studies have reported increased plant chlorophyll concentration when biostimulants were used. Cannabis plants fertilized with two different rates of a PGPR had significant higher SPAD (chlorophyll meter) values when comparing with the control (Pagnani et al., 2018).

Russo and Berlyn (1991) tested the combined effects of HA, marine algae (MA) and a non-hormonal reductant plant metabolite + B vitamins (Metab) on chlorophyll content of rye-grass (*Lolium perenne* L.). Their findings showed that HA + MA application yielded 74% more chlorophyll than the control and that combining HA + MA + Metab yielded 207% more than the control. On the other hand, Baldotto et al. (2009) tested HA from several sources on pineapple (*Ananas comosus* L.) chlorophyll estimates and reported no differences from the control.

However in the same study, Baldotto et al. (2009) detected an 11.54% difference in chlorophyll a and b ratio of one HA treatment compared to the control.

atLEAF values for treatments combining Humic + Biofertilizers were greater than the control, however they were similar to the treatments receiving only HA or biofertilizer. Following the analogous approach of biofertilizer + HA integration, Ferrini and Nicese (2002) assessed the influence of two commercial biostimulants products on English oak (*Quercus robur* L.). Both products containing HA and different types of biofertilizers had greater SPAD values in two consecutive years in comparison to control (no biostimulant).

The atLEAF readings collected at 42 days PE (post water stress) likely indicate that biostimulants increased chlorophyll content relative to the control (Table 4). The HA are natural soil constituents and can positively affect water retention due their high exchange capacity (Kelting, 1997). Biofertilizers are also known to mitigate water stress of several plants (Berruti, Lumini, Balestrini, & Bianciotto, 2016; Ruiz-Lozano & Azcón, 1995). Zarabi, Alahdadi, Akbari, and Akbari (2011) report that a combination of biofertilizer with synthetic fertilizer increased corn yield and leaf area index under drought stress compared to synthetic fertilizer alone. In fact, studies have shown chlorophyll content reduction in when cotton (*Gossypium hirsutum*) (Massacci et al., 2008), blueberry (*Vaccinium myrtillus* L.) (Tahkokorpi, Taulavuori, Laine, & Taulavuori, 2007) and sunflowers (*Helianthus annuus* L.) (Kiani, Maury, Sarrafi, & Grieu, 2008) were exposed to water stress. Considering that water stress reduced chlorophyll content in many studies and that biostimulant application generally maintained greater atLEAF readings that the control in this study, it might infer another effect of these compounds.

Table 4. Effect of biostimulants treatments on cannabis atLEAF values collected at 33, 42 and 55 days post-emergence (PE).

Group	Category	Treatment	33 days PE		42 days PE		55 days PE	
			atLEAF, mg cm^2					
None	None	Control	0.0524	b†	0.0600	d	0.0636	e
Biostimulant	Biofertilizer	Microgeo (M) 1x	0.0680	a	0.0718	ab	0.0725	abc
		Microgeo (M) 2x	0.0693	a	0.0698	abc	0.0728	ab
		Microgro Bio (MB) 1x	0.0670	a	0.0659	c	0.0686	bcd
		Microgro Bio (MB) 2x	0.0680	a	0.0655	c	0.0696	bcd
	Humic	Microlife Humic (H) 1x	0.0680	a	0.0672	bc	0.0677	cde
		Microlife Humic (H) 2x	0.0686	a	0.0653	c	0.0674	de
	Humic + Biofertilizer	M + H	0.0749	a	0.0740	a	0.0758	a
		MB + H	0.0658	a	0.0682	bc	0.0708	abcd

† Means within a column followed by the same letter are not significantly different at the 0.05 probability level.

3.3 Photosynthetic Efficiency (F_v/F_m)

Photosynthesis-related parameters were measured to test physiological responses of cannabis plants to additional input of biofertilizers and/or HA. According to Chandra (2003), the ability of plants to be successfully resilient in different environments, including under stress conditions, is associated with an ability to maintain high photosynthetic efficiency.

At 42 and 55 days PE was consistently greater in treatments receiving biostimulants in comparison to control values (Table 5), as was the case with atLEAF readings (Table 4). According to Jat and Ahlawat (2006), inoculation with biofertilizers such as *Rhizobium*, can increase the development of photosynthetic organs in plants and consequently maximize the accumulation of photosynthates. The single and/or dual inoculation of biofertilizers (*Rhizobium*, *Azotobacter*, *Azospirillum*, phosphobacteria) raised the chlorophyll level on Blackgram (*Vigna mungo* L.) and it could be used as an indirect parameter to quantify photosynthetic rate

(Selvakumar, Lenin, Thamizhiniyan, & Ravimycin, 2009). The photosynthetic rate was enhanced on rice (*Oryza sativa* L.) when *Trichoderma* – based biofertilizer was applied (Doni et al., 2018). Veres et al. (2009) specifically addressed the changes of photosynthetic efficiency (Fv/Fm) under biofertilizer application and found that F_v/F_m was higher in the treatments containing reduced N supply + biofertilizer. Thus, the use of biofertilizer could compensate for the N deficiency. The photosynthetic efficiency was also significantly higher on passion fruit (*Passiflora edulis*) tested with biofertilizers derived from cattle manure (de Oliveira Freire, Dias, Cavalcante, Fernandes, & de Lima Neto, 2014). Doost, Sharifi, Farzaneh, and Panah (2019) tested the application of three different biofertilizers (*Nitrobacter*, *Pseudomonas* and *Azosprillum*) on canola (*Brassica napus* L.) and all produced significantly higher F_v/F_m values than control (no biofertilizer inoculation).

In our study there were few differences in photosynthetic efficiency among biostimulant products, excluding the control. In other words, the individual and combined application of biofertilizer and HA and the rates of each product did not reveal any product that was more effective than others. The fact that each biostimulant category has a different mechanism to mitigate stress, which were described previously, may explain these similar results.

Like the biofertilizer treatments, PE values for the two application rates of HA and the combination with biofertilizers were generally greater than the control but very few differences existed among them. The application of HA alone and in combination with seaweed extract (SWE) was tested on creeping bentgrass (*Agrostis stolonifera* L.) during two years (Zhang, Ervin, & Schmidt, 2003a). In the first year, they found 18% and 15% increases in F_v/F_m values,

when HA or HA + SWE were applied, respectively. In the second year, the F_v/F_m values increased by 11% and 12%, respectively. A foliar application of HA + SWE also significantly increased F_v/F_m values on field- grown tall fescue (*Festuca arundinacea* Scheb.) (Zhang, Ervin, & Schmidt, 2003b). Interestingly, the presence of HA prevented the reduction of F_v/F_m values in duckweed (*Lemna gibba* L.) exposed to anthracene photoinduced toxicity (Gensemer, Dixon, & Greenberg, 1999). In fact, photosystem II (PSII) is anthracene's site of action (Huang, McConkey, Babu, & Greenberg, 1997) and PE is a measurement of PSII activity (Oxborough & Baker, 1997).

Shortly after drought stress was imposed (42 days PE) we observed that F_v/F_m values were greater than the control for all biostimulants treatments (Table 5). According to Richmond (1999), photosynthesis is the first plant physiological attribute to react to stress. There are a vast number of studies where the use of biofertilizers and/or HA improved photosynthetic efficiency values on plants under stress. The use of a biofertilizer derived from arbuscular mycorrhizal fungi (AMF) increase PE values 45 days after rice plants were subjected to water stress (Ruiz-Sánchez et al., 2010). Lotfi, Gharavi-Kouchebagh, and Khoshvaghti (2015) tested the effects of HA on rapeseed (*Brassica napus* L.) under well-watered, moderate and severe water stress. They found that HA application increased F_v/F_m values in all scenarios, however differences were greater under severe drought. An in-vitro test on potatoes reported that the application of potassium humate increased F_v/F_m values under water stress (Hassanpanah, 2009) but F_v/F_m values were similar to the control under normal conditions.

Water stress can increase secondary metabolites (Gorelick & Bernstein, 2014). Secondary metabolites are linked with the production of cannabinoids which can affect final yield of CBD or THC depending on the goals of the producer. Therefore, if the objective is to increase CBD or THC production, exposing cannabis plants to stress might be considered beneficial and the application of products reducing stress could harm CBD or THC production. For instance, moderate drought stress boosted the production of rosmarinic, ursolic, and oleanolic acid in heal-all (*Prunella vulgaris* L.) (Chen, Guo, Liu, Liao, & Zhu, 2011). Specifically addressing cannabis, there is evidence that moisture stress is linked with higher tetrahydrocannabinol content (THC) (Paris, Boucher, & Cosson, 1975). A similar phenomenon was reported by Murari, Puccini, Sanctis, and Lombardi (1983), where drier continental environments promoted the production of THC in cannabis plants naturally lacking in THC. Sharma (1975) reported a positive correlation between trichome density and low humidity environments in cannabis plants. Trichomes are important sites of CBD storage (Mahlberg & Kim, 2004), so this could affect final CBD yield.

Mechanistic evidence of how biofertilizers, HA and the combination of both sources maximize photosynthetic efficiency or even chlorophyll content is still developing. However, we speculate that the hormonal-like effects and antioxidant status of these compounds might have a causative role, especially under stress. External input of man-made hormone substances such as Indole-3-acetic acid (IAA) (Aldesuquy, 2000) and cytokinin (Noodén & Leopold, 2012) has increased photosynthetic rates. Vessey (2003) mentioned the presence of phytohormones or the precursor of hormones in many biofertilizers, including IAA produced by PGPR (Barazani & Friedman, 1999). It is unclear whether HA liberates locked hormonal compounds (chelating capability) or stimulates the production of hormones by microorganisms (du Jardin, 2012). In a

study testing the inoculation of PGPR on basil (*Ocimum basilicum* L.) under water deficit conditions, Heidari and Golpayegani (2012) concluded that the biofertilizer might improve antioxidant status. The addition of HA compound in a corn hydroponic production system induced positive changes in an important antioxidant (phenylpropanoid) pathway (Schiavon et al., 2010). The use of HA also improved the production of antioxidants such as phenolics and flavonoids in pomegranate (*Punica granatum* L.) (Anari Anaraki, Ghasem-Nejad, & Meyghani, 2016) and chicory (*Cichorium intybus* L.) (Gholami, Saharkhiz, Fard, Ghani, & Nadaf, 2018). As mentioned above there are studies reporting that plants exposed to stress will increase the production of secondary metabolites and potentially CBD or THC, so the use of biostimulants that minimize stress would decrease the production of these cannabinoids. There are also studies showing that biofertilizers and HA stimulate plant hormone production and consequently higher secondary metabolites, which is linked to greater cannabinoid concentration in plants. The application of biostimulants on cannabinoid concentration could be either positive or negative. More studies specifically addressing the effects of these biostimulants on cannabinoids would help to identify the pros and cons of biofertilizer and HA on CBD and THC production.

Table 5. Effect of biostimulant treatments on cannabis photosynthetic efficiency values collected at 33, 42 and 55 days post-emergence (PE).

Group	Category	Treatment	33 days PE		42 days PE		55 days PE	
			Fluorometer, F_v/F_m					
None	None	Control	0.526	b†	0.624	c	0.675	c
Biostimulant	Biofertilizer	Microgeo (M) 1x	0.642	a	0.707	b	0.753	ab
		Microgeo (M) 2x	0.657	a	0.710	b	0.766	a
		Microgro Bio (MB) 1x	0.622	a	0.685	b	0.717	bc
		Microgro Bio (MB) 2x	0.608	a	0.685	b	0.725	ab
	Humic	Microlife Humic (H) 1x	0.601	a	0.678	b	0.727	ab
		Microlife Humic (H) 2x	0.594	ab	0.685	b	0.723	ab
	Humic + Biofertilizer	M + H	0.664	a	0.748	a	0.756	ab
		MB + H	0.637	a	0.696	b	0.753	ab

† Means within a column followed the same letter by sites are not significantly different at the 0.05 probability level.

3.4 Shoot, Root and Total Biomass

Generally, shoot, root and total biomass at the end of the experimental period were similar between biostimulant treatments and the control (Table 6). Previous research has also reported that application of AMF and PGPR biofertilizer to cannabis plants did not increase shoot biomass compared to the uninoculated treatments (Gryndler et al., 2008). The less frequent increased cannabis biomass growth in response to biofertilizer application in our study might be due a plant etiolation, thus the biofertilizers promoted taller plants but biomass did not follow the same trend. Also, the biofertilizer effects on cannabis height were more evident in the two earlier data collection periods (33 and 42 days PE), then at 55 days PE, the height values were more similar to control which may explain fewer statistically significant values of biomass. Studies have tested the effectiveness of biofertilizers containing different strains of microorganism and the responses are variable depending on the active strains, harvest date, environment and growth parameter evaluated (Germida & Walley, 1996; Requena, Jimenez, Toro, & Barea, 1997). The

supplementary use of HA in addition to common fertilization practices for medical cannabis were tested by Bernstein, Gorelick, Zerahia, and Koch (2019) and they did not find differences in shoot biomass when HA was applied. There are a limited number of studies specifically addressing cannabis biomass and fertilization. According to Papastylianou, Kakabouki, and Travlos (2018) cannabis biomass is very responsive to N input. Aubin et al. (2015) tested five different applications of N, P, K in three locations in Canada and reported that cannabis biomass was more responsive to increased N input when comparing to P and K throughout all locations.

Only the application of Microgro Bio + Microlife Humic (MB + H) consistently produced greater shoot, root and consequently total biomass than the control (Table 6). Olivares, Aguiar, Rosa, and Canellas (2015) compared the effects of the combined use of biofertilizer + HA and biofertilizer alone applied to tomatoes (*Solanum lycopersicum* L.). Their study was started in the greenhouse where the growth substrate of tomato seedlings received the treatments, then a foliar application in the subsequent field trial. The combined use of biofertilizer + HA promoted higher shoot, root and fruit biomasses. However, in our study only one of two treatments combining biofertilizer + HA had greater biomass than the control. Plant biomass in response to Microgeo + Microlife Humic (M + H) did not differ from control.

The optimum rate of vermicompost-derived HA maximized hypocotyl and radicle mass of cannabis when compared to control (deionized water) (Ievinsh et al., 2017). Eyheraguibel, Silvestre, and Morard (2008) tested the effects of HA on corn cultivated under hydroponic conditions and found greater root, shoot and leaf biomass compared to non-treated plants. They concluded that this biomass increase could be the result of greater water use efficiency and

nutrient uptake resulting from HA application. In addition to hormone-like effects, Trevisan et al. (2011) also cited gene transcription and protein regulation as HA mechanisms of action.

Table 6. Effects of biostimulant treatments on cannabis shoot, root and total biomass values collected at 55 days post-emergence (PE).

Group	Category	Treatment	Shoot		Root		Total	
			Biomass, g					
None	None	Control	6.34	b†	2.18	b	8.52	b
Biostimulant	Biofertilizer	Microgeo (M) 1x	9.78	a	4.08	ab	13.86	a
		Microgeo (M) 2x	9.38	ab	3.50	ab	12.88	ab
		Microgro Bio (MB) 1x	8.12	ab	3.38	ab	11.50	ab
		Microgro Bio (MB) 2x	8.12	ab	3.78	ab	11.90	ab
	Humic	Microlife Humic (H) 1x	9.64	ab	4.34	ab	13.98	a
		Microlife Humic (H) 2x	8.28	ab	4.64	a	12.92	ab
	Humic + Biofertilizer	M + H	8.80	ab	3.62	ab	12.42	ab
		MB + H	10.34	a	4.50	a	14.84	a

† Means within a column followed by the same letter are not significantly different at the 0.05 probability level.

CONCLUSIONS

Application of biofertilizer and HA alone, or in combination, generally increased cannabis plant height, chlorophyll content and photosynthetic efficiency, especially immediately after a period of water stress (42 days PE). Cannabis biomass was generally not different from the control. However, total plant biomass at 55 days PE was greater than the control for application of Microgeo 1x, Microlife Humic 1x and 2x and Microgro Bioinoculant + Microlife Humic. Applying twice the labeled rate generally did not result in differences from the recommended rate. A range of rates would need to be tested in order to validate that the label rate is in fact the most appropriated application rate. There was not clear evidence of additional benefits from the combined application of biofertilizer + HA. However, the potential

biostimulant effects of these compounds that improve cannabis stress tolerance could prove important as it could impact CBD content. Further studies addressing the entire cannabis cycle should be conducted to validate these findings and confirm if these plant biostimulant effects extend to the end of the cannabis season. Previous literature has shown two contrasting sides of the use of these biostimulants on cannabis, where the greater stress tolerance provided by biofertilizer and HA could decrease the production of secondary metabolites and consequently CBD and THC concentration. Alternately, plant biostimulants such as biofertilizer and HA acting on plant hormonal production could increase secondary metabolites and CBD and THC. Therefore, the next step for this research would be testing the effects of these compounds on CBD and THC content.

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Chapter 4: An Integrated Plant Nutrition System (IPNS) for corn in Mid-Atlantic USA

ABSTRACT

Current trends in agriculture have moved towards more sustainable cultivation systems with higher efficiency of input use. A variety of materials, derived from different resources, can serve as a crop nutrient source. An Integrated Plant Nutrition System (IPNS) uses the combined and harmonious use of inorganic, organic and biological nutrient resources to maximize efficiency of inputs. We evaluated the effects of commercial nitrogen (N) fertilizer, humic acid compounds (HA), compost/manure teas and bioinoculants as inorganic, organic and biological resources, respectively and their synergy over three years on corn (Zea mays L.) in the Mid-Atlantic USA. The individual and combined application of HA and biofertilizer following the IPNS influenced corn height and leaf greenness to varying degrees, most likely due to biostimulant effects. In 2017, corn height, NDVI, greenness and vigor responded positively to biostimulant application to varying magnitudes and growth stages, however grain yield and nutrient content were not affected. In combined studies from 2018 and 2019 corn height was not impacted by biostimulant application but NDVI, photosynthetic efficiency, greenness and vigor were increased at different doses and corn growth stages. The combined use of HA + biofertilizer (Microlife Humic + Microgeo) was the only treatment leading to increased grain yield. This study demonstrates that the individual and combined application of HA and biofertilizer can influence corn growth and vigor at various points during the growing season. However, the current study cannot conclusively affirm that the integrated use of HA and biofertilizers (IPNS) is a better practice than the application of each compound individually.

Keywords: biostimulant, humic acid, biofertilizer, manure tea, compost tea, humates

INTRODUCTION

To date, most agricultural research has prioritized yield increases at the expense of crop quality or optimization of resources (Bulgari et al. 2015). However, current trends in agriculture have moved towards more sustainable cultivation systems with higher efficiency of input use, since mineral nutrient losses due to runoff, leaching, erosion and gas emissions are leading to environmental degradation (Adesemoye and Kloepper 2009, Bhattacharjee, Singh, and Mukhopadhyay 2008, Tilman 1998). Moreover, the excessive use of chemical fertilizer can decrease soil fertility and some agricultural systems are approaching theoretical maximum yield, where further fertilizer input does not result in increased yield (Ahmed 1995). Besides approaching theoretical maximum yield and negative environmental impacts, the three macronutrients nitrogen (N), phosphorus (P) and potassium (K) are produced by the utilization of limited resources such as mining or fossil fuel consumption and the growing world population is increasing the total use of fertilizers as a consequence of higher demand for food (Frink, Waggoner, and Ausubel 1999, Vitousek et al. 1997). This pressure on limited resources necessitates the promotion of practices that maximize fertilizer efficiency and the scientific community is increasingly focused on this topic. According to Adesemoye and Kloepper (2009) just as research in the last century focused on reducing the use of pesticides, this century will be target ways to decrease the use of fertilizers.

A huge variety of materials can serve as a crop nutrient sources and they can be derived from inorganic, organic and biological reserves. Thereby, the atmospheric deposition, biological

activity, plants residues, organic manure, urban waste and synthetic fertilizer are potential ways to enlarge the natural nutrients reserves already occurring in the soil. (Aulakh and Grant 2008). According to the Food and Agriculture Organization of the United Nations (FAO) (Shand 2007), the definition of an Integrated Plant Nutrition System (IPNS) is "the adaptation of the plant nutrition and soil fertility management in farming systems to site characteristics, taking advantage of the combined and harmonious use of inorganic, organic and biological nutrient resources to serve the concurrent needs of food production and economic, environmental and social viability." An IPNS requires an understanding of nutrient dynamics throughout the soil-microbe-plant system as nutrient availability is interconnected and can address issues ranging from nutrient excess to nutrient depletion and (Aulakh and Grant 2008).

The IPNS is a broad term, thus the combined practices need to explore the effects of different resources (Snapp, Mafongoya, and Waddington 1998). Under the category of inorganic, organic and biological there are many products or compounds and consequently the synergy of these products can vary depending on the strategy of the producer. According to the IPNS definition, the most effective use of the prescribed compounds used in an integrated manner will depend on their efficacy in a specific environment and local production systems (Aulakh and Grant 2008). The diversity of compounds and the potential negative or positive interaction of them in a particular ecosystem dictates the need for local research when developing the IPNS. Chemical or inorganic inputs can often be used more efficiently when combined with a proper organic/biological source (Adesemoye, Torbert, and Kloepper 2008). Thus, the challenge is to find the most appropriate combinations of organic and biological resources that result in positive synergy with inorganic fertilizers in the IPNS.

Humic acid (HA) is a heterogeneous and stable organic complex formed naturally in soils with the capability to give soil structure, porosity, water holding capacity, cation and anion exchange, and be part of the chelation of mineral elements (Pettit 2004). Moreover, the hormone-like activity present in HA can maximize plant nutrition along with the soil chelation function and increase plant growth (Vaughan and Malcolm 1985). Hence, the soil and plant effects can be intercorrelated and complementary, for instance, the plant root growth stimulation and the soil cation exchange capacity can be enhanced by HA (Chen and Aviad 1990). In other words, greater root area (Baldotto and Baldotto 2014) and nutrient accessibility (Chang et al. 2012) can stimulate plant development. Other parameters such as improved fruit quality and increased berry size in table grapes [*Vitis Vinifera* (L)], were reported as a beneficial result of HA application (Ferrara and Brunetti 2010).

Biofertilizers or bioinoculants are defined as substances derived from biological resources containing living microorganisms and used to maximize the uptake of inorganic nutrients and stimulate vegetative development by colonizing the interior of plants or the rhizosphere (Vessey 2003). They can be applied to seed, plant surfaces and soil (Sahu, Priyadarshani, and Rath 2012). The integrated use of these different biological compounds have shown to be promising in the functions mentioned in the definition above (Barea, Toro, and Azcón 2007, Hodge, Campbell, and Fitter 2001) plus to support the health of plants (Weller 2007). They can be used to decrease the deleterious impacts of chemical fertilizers (Bashan 1998). According to Goel et al. (1999) nitrogen fixing, phosphate solubilizing and plant growth promoting microorganisms exist in the biofertilizer category. Thus, the application of one or more beneficial bacteria and/or fungi has positively affected plant growth, yield and yield

components on corn (*Zea Mays* L.) (Zarabi et al. 2011, Yosefi et al. 2011) and common beans (*Vicia faba* L.) (El-Habbasha, Hozayn, and Khalafallah 2007). Furthermore, biofertilizer products developed from compost tea enhanced vegetative growth of corn, soybean (*Glycine Max* L.) and lettuce (*Lactuca sativa* L.), possibly by improving nutrient uptake (Kim et al. 2015).

Humic acid and biofertilizers belong to a broader category of biostimulants due the fact that they are substances and microorganisms that enhance plant growth, however this categorization is still evolving (Calvo, Nelson, and Kloepper 2014). Canellas and Olivares (2014) exposed the basic mechanisms and benefits of the integrated use of these two biostmulants on many crops. However the actions of microorganisms applied as biofertilizers in a heterogeneous soil can be complex due to the existence of competitors and predators inhabiting the same environment (Young et al. 2006). Since HA is a major part of soil organic matter (SOM) and influences soil properties, HA might support the acclimation of inoculated microorganisms from the biofertilizer application (Canellas et al. 2013). The HA influence on root growth and architecture (Nardi, Concheri, and Dell'Agnola 1996) may favor the interaction between microbes and roots. Furthermore, the addition of HA is known to induce changes in the the structure of soil microbial communities in the rhizosphere of corn (Puglisi et al. 2009).

A thorough understanding of plant–microbial associations is crucial to boost the efficacy of plant nutrient uptake (Peoples and Craswell 1992, Zhu et al. 2001) However, little work has been done regarding crop response to the combined effect of HA and biofertilizers (Rose et al. 2014) and we hypothesize that the synergetic effects of HA + biofertilizers can improve corn development. This research assessed the effects of the integrated and individual use of HA and

compost/manure teas and bioinoculants along with inorganic fertilizer on corn growth, vigor and yield, compared to a control receiving only inorganic fertilizer.

MATERIALS AND METHODS

2.1 Design of experiments and management

Experiments in Virginia were conducted in 2017 – 2019 at Kentland Farm in Blacksburg and in 2018 near Champlain, VA. Experiments were planted into soybean residue at Champlain and into corn stover in all years at Blacksburg with direct seeding methods. Five biostimulant products (including organic and biological resources) were tested in this study. The organic products were Monty's and MicroLife Humic Acid Complex®; and the biological products were SoilSoup®, Microgeo® and Microgro Supreme Bioinoculant®. The humic category was represented by Monty's® and MicroLife Humic Acid Complex® which was constituted of 2% humic acid / 1% organic carbon and 15% humic acid /1% fulvic acid, respectively. One of the three biological fertilizers was Microgeo® which is a Brazilian patented product categorized as a manure tea. This biofertilizer is composed of organic compounds, active and dormant cells from various microorganisms (bacteria, yeasts, filamentous fungi, and algae), metabolites and organo-mineral chelates and it is produced through continuous anaerobic fermentation in a liquid media (D'Andrea 2002). According to the technical manual, the preparation is using the CLC® (Continuous Liquid Composting) process, where 5% of the commercial biological fertilizer Microgeo ®, 15% of ruminal content and water are mixed in a tank exposed to a sunlight. After 15 days the biofertilizer is ready to be applied. SoilSoup® is an aerobic compost tea generated via fermentation of vermicompost over 24 hours with the addition of nutrient solution (molasses, bat guano, sea bird guano, soluble kelp, langbeinite, natural citric acid, ancient seabed minerals,

yucca) and oxygen to the system (aquarium pump).The Microgro Supreme Bioinoculant® is a water-soluble powder containing 76 strains of bacteria and fungi including 11 Mycorrhizal species and microbial food (sugars, humic acid, kelp, amino acids and yeast extract). All products are further described in Table 1.

Table 1. Products description and components

Resource	Category	Subcategory	Name	Components
Organic	Humate	Humic acid	Monty's Liquid Carbon	2% humic acid and 1% organic carbon derived from brown coal
			Microlife Humic Acid Complex	15% humic acid and 1% fulvic acid derived from leonardite
Organic + Biological	Biofertilizer	Manure tea	Microgeo	recancitrant substances, biodynamic preparations, pentoses, minerals and the microorganisms produced in the manure tea fermentation
		Compost tea	SoilSoup	molasses, bat guano, sea bird guano, soluble kelp, langbeinite, natural citric acid, ancient seabed minerals, yucca and the microorganisms produced in the compost tea fermentation
		Bioinoculant	Microgro Supreme Bioinoculant	76 different strains of bacteria and fungi placed on dry milk carrier loaded with microbial food. The microorganisms included are: species of Genus Bacillus, Psuedomonas, Streptomycetes, Trichoderma, and Endo and Ectomycorrhizal Fungi

One experiment was initiated in 2017 with two N sidedress rates of 107 and 134 kg N ha^{-1} applied as urea-ammonium nitrate (UAN, 30% N) in a factorial arrangement with the individual and combined use of three biostimulant products. These sidedress rates corresponded to 80 and 100% of the recommended sidedress N rate based on corn yield goal at the Blacksburg

location. The lack of a significant interaction between N rate and biostimulants led to a redesign of the experiment for 2018 and 2019. In the latter two years, a single N sidedress rate (107 kg N ha^{-1} in Blacksburg and 178 kg N ha^{-1} in Champlain) was homogeneously applied as urea-ammonium nitrate (UAN, 30% N) in all plots and five total biostimulants were tested independently and in combination. A detailed description of management practices is presented in Table 2 while treatment combinations used in different years are shown in Tables 3 and 4.

The experimental design in each case was a randomized complete block design with 12 treatments and four replications with a plot size of 3 m x 7.6 m and 0.61 m alley and corn planted in 76 cm rows. Corn hybrid, seeding rate, and field activities are presented in Table 2. Other management practices and inputs including weed and pest control and P and K application followed Virginia Cooperative Extension recommendations for corn production (Brann, Holshouser, and Mullins 2000).

Table 2. Field activities and corn hybrid listing for experiments at Blacksburg and Champlain VA, 2017 – 2019.

Field activity	Blacksburg			Champlain
Planting date	5/17/2017	5/16/2018	4/29/2019	4/23/2018
Corn Hybrid	Mid-Atlantic 8146 VT3P	Mid-Atlantic 8146 VT3P	Syngenta - NK1354	Hubner H4563
Population (plant ha^{-1})	62,500	62,500	62,500	88,900
Side dress N fertilization date	6/20/2017	6/11/2018	6/3/2019	6/7/2018
Harvest date	11/1/2017	10/23/2018	9/18/2019	9/4/2018

Application rates of the organic and biological compounds were consistent with label recommendations for those products. They were applied to soil shortly before emergence and to plant foliage at corn growth stages V4, V7 and V11. The application rates were: Microgeo

applied at 150 L ha^{-1} applied full strength to soil and 3% strength at V4, V7, V11; SoilSoup applied at 234 L ha^{-1}, full strength; Microgro Supreme Bio applied at 6.1 kg ha^{-1} diluted in 234 L of water; (2018 and 2019) and Monty's Liquid Carbon applied at 4.6 L ha^{-1} diluted in 234 L of water and Microlife Humic at 14 L ha^{-1} diluted in 234 L of water (2018 and 2019).
At V5, V10 and VT, plant height, plant greenness (ranked from 0-10, personal visual evaluation where 0 was less green and 10 was darker green), plant vigor (visual assessment from 0-10, with 0 showing very poor vigor and 10 indicating greatest vigor), normalized vegetation index (NDVI) collected using a Greenseeker® model 505 handheld sensor unit (Trimble Navigation, Sunnyvale, CA) used according to the methodology of Govaerts and Verhulst (2010) and photosynthetic efficiency measured with an OS-50II fluorometer (Opti-Sciences, Tyngsboro, MA) (2018-2019). Corn was harvested from the center two rows of each plot using a Kincaid 8X (Kincaid Equipment Manufacturing, Haven, KS) small plot combine equipped with GrainGage system measuring total weight, moisture and test weight of each plot (Juniper Systems Inc., Logan, UT). A subsample of grain was collected to determine grain moisture using a Dickey-John (Dickey-John, Auburn, IL) and grain yield was adjusted to 155 g kg^{-1} moisture content. Grain subsamples were also analysed for starch and protein content using near infrared spectroscopy (FOSS XDS, Eden Prairie, MN).

Analysis of variance was conducted using PROC GLIMMIX in SAS (SAS Institute, 2017) to evaluate treatment effects on plant height, vigor, greenness, NDVI, photosynthetic efficiency (PE), grain yield and grain nutrient content. Biostimulant products and N rates were considered fixed effects, while experimental site, year and replication were considered random effects. In 2017, we initially screened the data for potentially important biostimulant by N rates

interactions, but no significant interactions were found. Thus, our final statistical model for 2017 averaged over N rates. In the absence of biostimulant, location and year interactions in 2018 and 2019, statistical results were pooled over locations and years. In all years, mean separation was conducted using the Dunnett's option within the LSMEANS statement when F-tests indicated that significant differences existed ($p<0.05$) due to treatments.

Table 3. Treatments arrangement during the year of 2017 in Blacksburg.

No.	Treatments 2017			
	Subcategory	Product Name and Abbreviation	Label (L/ha)	UAN (kg/ha)
1		Control (C)	0	107
2	Humic	Monty's (H)	4.6	107
3	Biofertilizer	Microgeo (M)	150	107
4		SoilSoup (S)	234	107
5	Humic + Biofertilizer	Monty's + Microgeo (H + M)	4.6 and 150	107
6		Monty's + SoilSoup (H + S)	4.6 and 234	107
7		Control (C)	0	134
8	Humic	Monty's (H)	4.6	134
9	Biofertilizer	Microgeo (M)	150	134
10		SoilSoup (S)	234	134
11	Humic + Biofertilizer	Monty's + Microgeo (H + M)	4.6 and 150	134
12		Monty's + SoilSoup (H + S)	4.6 and 234	134

Table 4. Treatments arrangement in Blacksburg (2018 – 2019) and Champlain (2018).

No.	Treatments 2018 - 2019		
	Subcategory	Product Name and Abbreviation	Label Rate
1		Control (C)	0
2	Humic	Monty's (H)	4.6 L ha^{-1}
3		Microlife Humic (LH)	14 Lha^{-1}
4	Biofertilizer	Microgeo (M)	150 L ha^{-1}
5		SoilSoup (S)	234 L ha^{-1}
6		Microgro Supreme Bio (B)	6.1 kg ha^{-1}
7	Humic + Biofertilizer	Monty's + Microgeo (H + M)	4.6 L ha^{-1} / 150 L/ha^{-1}
8		Monty's + SoilSoup (H + S)	4.6 L ha^{-1} / 234 L ha^{-1}
9		Monty's + Microgro Supreme Bio (H + B)	4.6 L ha^{-1} / 6.1 kg ha^{-1}
10		Microlife Humic + Microgeo (LH + M)	14 L ha^{-1} / 150 L ha^{-1}
11		Microlife Humic + SoilSoup (LH + S)	14 L ha^{-1} / 234 L ha^{-1}
12		Microlife Humic + Microgro Supreme Bio (LH + B)	14 L ha^{-1} / 6.1 kg ha^{-1}

Results and discussion

3.1 *Growing season environmental conditions*

Monthly average temperatures in the 2017 growing season in Blacksburg were above the 30 year average the entire growing season, April – October (Figure 1). The monthly average temperature in April was 5.5°C higher than the 30 year average, favouring early season corn growth and development. Rainfall was above normal in April, August and October, and considerably above normal in May. Below-normal rainfall occurred in June and September. The 2018 – 2019 season in Blacksburg and Champlain presented temperature and rainfall values that were above normal in most of the season (Figure 1 and 2) except in Blacksburg in 2019, where the rainfall was lower than the 30 year average in May, August and September.

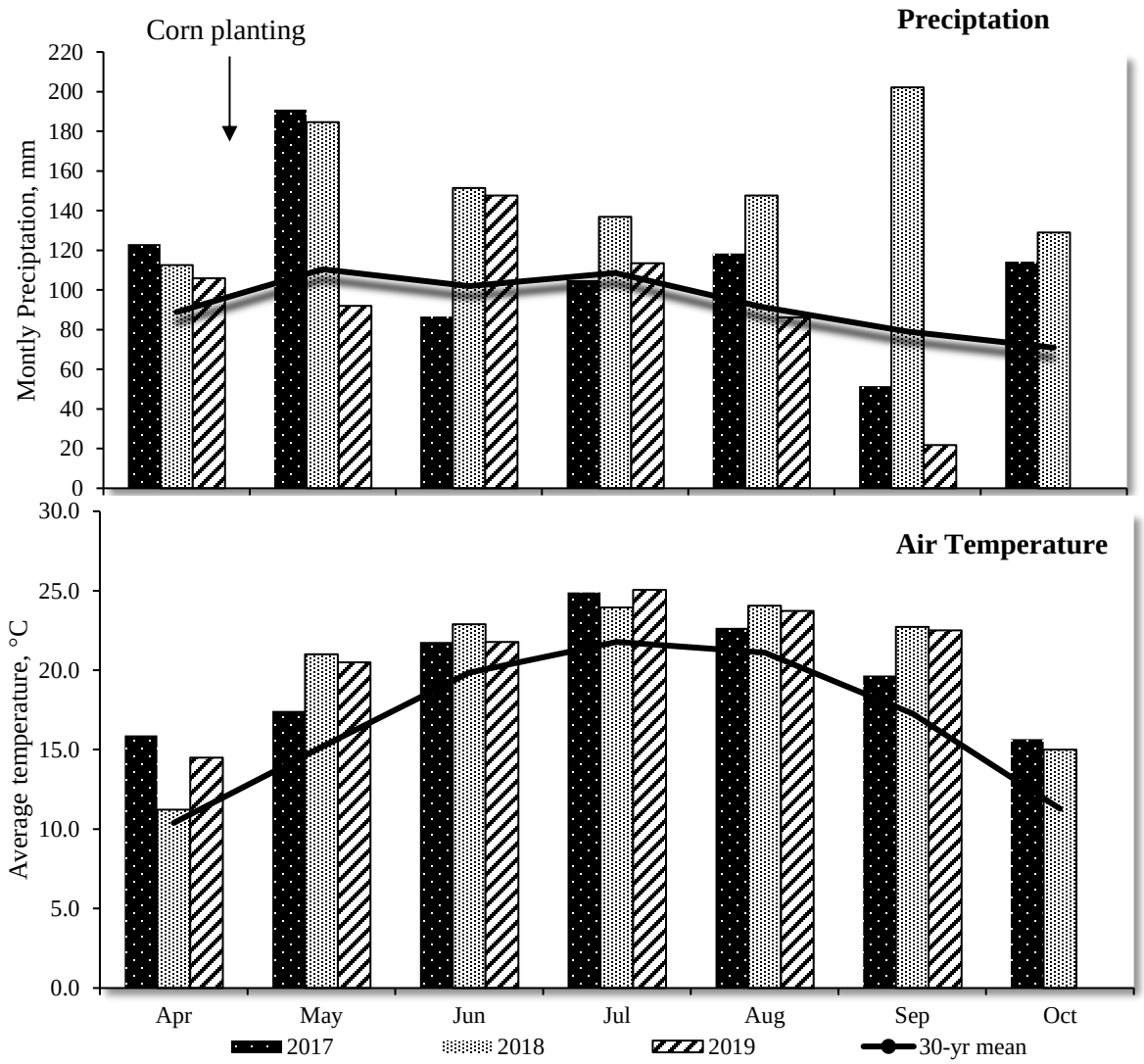

Figure 1. Average total monthly precipitation and monthly air temperature for the 2017 - 2019 growing seasons in Blacksburg – VA area. Data were collected at National Oceanic and Atmospheric Administration National Climatic Data Center (NOAA) website - https://www.ncdc.noaa.gov/cdo-web/ (accessed 1 Oct. 2019). Circular symbols connected by lines represent normal temperature and precipitation recorded from 1981–2010 in Virginia.

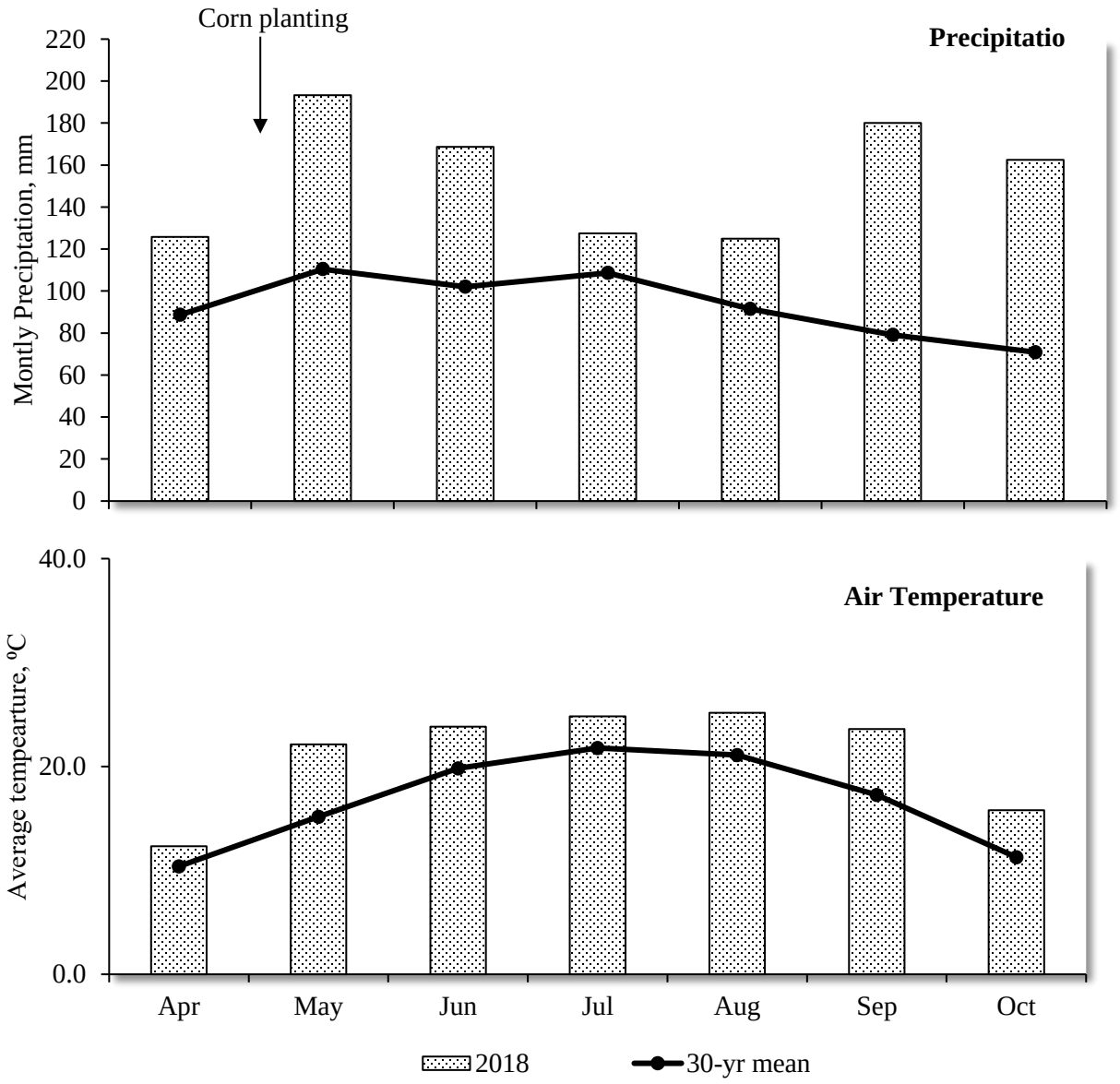

Figure 2. Average total monthly precipitation and monthly air temperature for the 2018 growing season in Champlain – VA area. Data were collected at National Oceanic and Atmospheric Administration National Climatic Data Center (NOAA) website - https://www.ncdc.noaa.gov/cdo-web/ (accessed 1 Oct. 2019). Circular symbols connected by lines represent normal temperature and precipitation recorded from 1981–2010 in Virginia.

3.2 *Year 2017*

Analysis of variance found no significant interaction between N rates. Therefore, we combined the results obtained in both N rates for each biostimulant treatment (Table 5).

Table 5. Effects of the biostimulant treatments on corn height, NDVI, greenness and vigor, collected at V5, V10 and Tasselling growth stage in Blacksburg, 2017.

Growth stage	Subcategory	Biostimulant treatment	Difference between treatments and control values							
			Height, cm		NDVI		Greenness, rank †		Vigor, rank ‡	
V5	Humic	Monty's	5.3	*	0.026		0.6		0.6	
	Biofertilizer	Microgeo	6.2	*	0.060	*	1.0	*	0.8	*
		SoilSoup	5.0	*	0.001		0.5		0.3	
	Humic + Biofertilizer	Monty's + Microgeo	8.5	*	0.013		0.6		0.5	
		Monty's + SoilSoup	8.2	*	0.012		0.2		0.4	
		Actual Control Values	66.2		0.628		8.1		8.3	
V10	Humic	Monty's	2.0		0.030		1.0	**	0.8	**
	Biofertilizer	Microgeo	2.2		0.049		0.8	**	0.6	
		SoilSoup	1.6		0.031		0.3		0.5	
	Humic + Biofertilizer	Monty's + Microgeo	3.6		0.063	**	1.1	**	0.7	**
		Monty's + SoilSoup	1.8		0.049		0.9	**	0.6	
		Actual Control Values	138.6		0.710		8.6		9.0	
Tasselling	Humic	Monty's	-5.6		0.036	***	1.0	***	0.7	***
	Biofertilizer	Microgeo	1.8		0.034	***	1.0	***	0.6	***
		SoilSoup	-1.8		0.023		0.7	***	0.6	***
	Humic + Biofertilizer	Monty's + Microgeo	1.0		0.033	***	1.1	***	0.7	***
		Monty's + SoilSoup	-2.2		0.032	***	0.9	***	0.6	***
		Actual Control Values	268.5		0.789		8.8		9.2	

*, **, *** Means separation within each growth stage are significantly different as $\alpha<0.05$.
† visual assessment where 0 was less green and 10 was darker green.
‡ visual assessment where 0 showing very poor vigor and 10 indicating greatest vigor.

Plant height showed statistically significant differences only at the V5 growth stage, where all biostmulant treatments resulted in taller plants than the control. The use of HA and

biofertilizers alone and in combination stimulated corn height similarly at V5. Previous work has demonstrated that increasing doses of HA applied directly to the soil (Daur and Bakhashwain 2013) and foliar application (Gaikwad et al. 2012) increased corn height, possibly due the mitigation of stress. Foliar HA application has resulted in greater corn height mainly under water deficit, but has been reported under full irrigation as well (Moghadam, Khamene, and Zahedi 2014, Abdulameer and Ahmed 2019). These four studies measured differences in corn height around tasseling but we found no differences at this stage in our studies. Also, these studies reported increased grain yield or dry biomass when HA was applied which was not observed in our study in 2017. Regarding the influence of HA on corn at early vegetative stages, Sun et al. (2016) found that different concentrations of HA increased corn seedling height under hydroponic conditions. Use of HA has also influenced height of other plant species such as millet (*Panicum miliaceum* L.) (Kuşvuran and Babat 2011), canola (*Brassica Napus* L.) (Sani 2014) and cucumber (*Cucumis sativus* L.) (El-Nemr et al. 2012). Various methods of HA application to plants have shown its influence on cell elongation (Vaughan 1974), nutrient uptake (Ayas and Gulser 2005), improved soil structure and water retention (Lobartini, Orioli, and Tan 1997) and hormone-like effects (Canellas et al. 2011). Thus, all these factors may contribute to increased corn height well as greater NDVI, greenness and vigor (Table 5). Furthermore, the application of HA to corn has increased dry biomass and forage harvest index and decreased the need for P fertilizer (Rezazadeh, Khorasani, and Haghighi 2012). In the current experiment, N fertilizer was the only macronutrient variable tested and the interaction HA was not significant. Varanini and Pinton (1995) stated that N release is influenced by humic substances but other soil factors such as N mineralization and microbial activity should be taken into consideration.

The biofertilizer treatments Microgeo and SoilSoup performed similarly in terms of corn height, with values that were statistically greater than the control in the individual applications and in combination with HA, but only at the V5 growth stage. Under greenhouse conditions an increasing dose of a bovine biofertilizer made in a very similar manner as the biofertilizer Microgeo, consistently increased corn height at 60 days after sowing , most likely due the presence of nitrogen in the biofertilizer (Viana et al. 2014). Regarding the integrated use of synthetic, organic and biological resources, studies addressing baby corn (Jinjala et al. 2016, Dadarwal, Jain, and Singh 2009), sweet corn (Mukhlis and Lestari 2014) and corn (Singh et al. 2018) reported that combining synthetic fertilizer + biofertilizer resulted in taller plants than the application of each resource alone. Moreover, Baloach et al. (2014) revealed that the application of biofertilizer alone and in combination with HA increased corn height when compared to the untreated control. Again, the height values in these corn studies previously mentioned were collected at tasselling and in the data from 2017 the biofertilizer treatments presented significant values only at V5 (Table 5). Mobasser and Moradgholi (2012) revealed that corn plant height and yield can vary substantially due to interaction between different biofertilizers strains and corn hybrids. Studies have shown that numerous types of biofertilizers have the potential to act on plant height, as a result of different mechanisms, such as nitrogen fixation in legumes and non-legumes (Dobereiner and Pedrosa 1987), solubilization of phosphates, micronutrient and minerals (Goldstein and Liu 1987, Wu et al. 2005), plant defence against biotic and/or abiotic stress (Mastouri 2010) and stimulation of plant growth regulators like auxins, gibberellins, and cytokinin (Contreras-Cornejo et al. 2009). These mechanisms might help to explain the positive effects of biofertilizers on NDVI, greenness and vigor as well.

The application of HA alone significantly increased NDVI values only at tasselling, but the combination HA + biofertilizer resulted in NDVI values significantly higher than control at V10 and Tasselling (Table 5). Zhu and Li (2018) compared the application of two rates of HA against the control (without HA) on turf grass in Colorado and North Dakota and found that HA significantly increased NDVI values at both rates and locations. Also, when comparing to control, HA applications resulted in higher NDVI from May through September, but this was only measured at V10 in 2017. Values of NDVI can be associated with chlorophyll concentration because reflectance of the specific wavelengths of light used in the equation to derive NDVI are very similar to those associated with production of chlorophyll (Ciganda, Gitelson, and Schepers 2009). According to Pishchik et al. (2016), the synergistic effect of HA + biofertilizer increased chlorophyll concentration of lettuce compared to the application of each compound alone.

The biofertilizer Microgeo had greater NDVI than SoilSoup with values greater than the control in the individual application at V5 and tasselling as well as in combination with HA at V10 and tasselling (Table 5). Viana et al. (2014) tested a liquid biofertilizer derived from bovine manure similar to Microgeo and the increasing doses caused significantly higher photosynthetic rates than the control. SoilSoup increased NDVI over the control only in combination with HA at tasselling (Table 5). The integrated use of two biofertilizers, where one of them contained HA, has statistically increased NDVI values of corn under deficit and normal irrigation (Shirkhani and Nasrolahzadeh 2016). Kazi et al. (2016) stated that while the application of biofertilizer did not significantly affect NDVI on wheat (*Triticum aestivum* L.), numerically higher values were found in inoculated plots compared to the uninoculated control. This may be similar to the

observations in this experiment, where few treatments had NDVI values statistically higher than the control, however all the values were numerically higher.

Spectral reflectance (NDVI) can be an important indicator of stress for corn (Wang, Cherkauer, and Bowling 2016), which can be correlated to the visual quality parameters collected in this experiment (greenness and vigor). The treatments containing HA alone and HA + biofertilizer showed significantly higher greenness and vigor values at V10 and tasselling. The same study mentioned previously from Zhu and Li (2018), showed that the highest application rate of HA consistently had higher values of visual turf quality than the control from May through September in Colorado and North Dakota. In this case, the turf grass visual quality assessment followed the (Morris and Shearman 2000), which included color and plant density as criteria to grade the treatments. These two parameters were used to evaluate greenness and vigor, respectively, in this current experiment. de Melo, Baldotto, and Baldotto (2015) also found that corn responded positively to the application of HA and enhanced the initial corn vigor and crop's yield potential.

The biofertilizers alone and in combination with HA generally resulted in higher scores for greenness and vigor at V10 and tasselling (Table 5). The fact that most of the treatments presented numerically higher values than control in terms of height and NDVI might have impacted the visual grading for greenness and vigor.

The other plant parameters collected in this experiment such as grain yield and nutrient content were not statistically influenced by the treatments.

3.3 Year 2018 and 2019

In Blacksburg in 2018 and 2019 and Champlain in 2018 80% of the recommended N rate was consistently applied in all field trials. Furthermore, due to the lack of interaction, we combined the data from the two years in Blacksburg and one year in Champlain for each treatment (Table 6).

Table 6. Effects of the biostimulant treatments on corn NDVI, Photosynthetic Efficiency (PE), greenness and vigor, collected at V5, V10 and tasselling growth stage in Blacksburg, 2018 and 2019 and Champlain, 2018.

Growth stage	Subcategory	Biostimulant treatment	Difference between treatments and control values							
			NDVI		PE, Fv/Fm		Greenness, rank †		Vigor, rank ‡	
V5	Humic	Monty's	0.014		0.0175		0.41		0.09	
		Microlife Humic	0.038		0.0884	*	0.66	*	0.44	
	Biofertilizer	Microgeo	0.046		0.0860	*	0.72	*	0.50	
		SoilSoup	0.024		0.0501	*	0.69		0.31	
		Microgro Bioinoculant	0.037		0.0876	*	1.03	*	0.56	
	Humic + Biofertilizer	Monty's + Microgeo	0.064	*	0.1038	*	0.84	*	0.63	*
		Monty's + SoilSoup	0.023		0.0816	*	0.41		0.13	
		Monty's + Microgro B.	0.042		0.0920	*	0.84	*	0.63	*
		Microlife Humic + Microgeo	0.091	*	0.1146	*	0.84	*	0.63	*
		Microlife Humic + SoilSoup	0.046		0.0908	*	1.03	*	0.69	*
		Microlife Humic + Microgro B.	0.050		0.0898	*	0.91	*	0.56	*
		Actual Control Values	0.404		0.4849		8.72		9.06	
V10	Humic	Monty's	0.010		0.0166		0.41		0.31	
		Microlife Humic	0.088	**	0.0566	**	0.69		0.53	
	Biofertilizer	Microgeo	0.076		0.0531	**	0.63		0.47	
		SoilSoup	0.001		0.0146		-0.09		0.00	
		Microgro Bioinoculant	0.101	**	0.0475	**	1.00	**	0.69	
	Humic + Biofertilizer	Monty's + Microgeo	0.066		0.0683	**	0.81		0.56	
		Monty's + SoilSoup	0.040		0.0423	**	0.41		0.25	
		Monty's + Microgro B.	0.048		0.0408	**	0.06		-0.03	
		Microlife Humic + Microgeo	0.092	**	0.0606	**	0.80	**	0.46	
		Microlife Humic + SoilSoup	0.086		0.0548	**	0.77		0.69	
		Microlife Humic + Microgro B.	0.111	**	0.0733	**	0.94	**	0.59	
		Actual Control Values	0.627		0.6407		8.41		8.75	
Tasselling	Humic	Monty's	0.011		-0.0004		-0.19		-0.16	
		Microlife Humic	0.025		0.0116		0.53		0.43	
	Biofertilizer	Microgeo	0.026	***	0.0153	***	0.06		-0.04	
		SoilSoup	0.021		0.0080		0.22		0.12	
		Microgro Bioinoculant	0.008		0.0086		0.34		0.31	
	Humic + Biofertilizer	Monty's + Microgeo	0.023		0.0205	***	0.13		-0.04	
		Monty's + SoilSoup	0.020		0.0072		0.09		-0.01	
		Monty's + Microgro B.	0.019		0.0056		0.03		-0.01	
		Microlife Humic + Microgeo	0.029	***	0.0353	***	0.28		0.18	
		Microlife Humic + SoilSoup	0.004		0.0146		0.50		0.40	
		Microlife Humic + Microgro B.	0.013		0.0235	***	0.31		0.21	
		Actual Control Values	0.799		0.802		9.44		9.54	

¥ The data from 2018 and 2019 were combined due the lack of significance (treatments x growth stages x years interaction).
*, **, *** Means separation within each growth stage are significantly different as $\alpha<0.05$.
† visual assessment where 0 was less green and 10 was darker green.
‡ visual assessment where 0 showing very poor vigor and 10 indicating greatest vigor.

No effect of treatment on corn height was observed in studies in 2018 and 2019. El-Mekser, Mohamed, and Ali (2014) also found non-positive effects of HA on corn height when considering different locations and growing seasons. Similarly, habanero pepper (*Capsicum chinense*) did not show increased plant height in response to application of biofertilizer (Moreno-Salazar et al. 2019).

In 2017, we observed significant effect of Monty's HA application on NDVI at tasseling. However, in later years Monty's HA did not affect NDVI while the other HA product, Microlife Humic had NDVI values greater than the control when applied alone at V10 and with biofertilizer at all growth stages. We followed the label rate of each product and this resulted in a greater application rate of the Microlife Humic product. This product also had a higher concentration of HA compared to Monty's. There is clear evidence that different HA application rates and methods can promote different magnitude of effects or basically no effect (Yildirim 2007, Liu, Cooper, and Bowman 1998). According to Senesi and Brunetti (1996), HA parameters such as: molecular heterogeneity, aromaticity and structural polycondensation, aliphatic character, oxygen presence and acidity can impact economic value, agronomic efficacy and environmental safety of the final HA product. None of the characteristics mentioned above are included in the HA products label and even the method to quantify the level humic substances percent is not specifically described. However, institutions such as the Humic Products Trade Association are trying to standardize the main HA specifications included in the

product label. Lamar et al. (2014) created a methodology for routine regulatory and industrial use to establish and verify contents of HA commercial products due the increasing use of humic substances in agriculture and the real necessity of farmers, consumers and regulators to obtain trustworthy information from the label.

As occurred in 2017, NDVI values were greater for the biofertilizer Microgeo than other biofertilizer treatments. In 2018-19, Microgeo alone increased NDVI values over the control at tasselling and in combination with Microlife Humic at all growth stages (Table 6). SoilSoup application did not result in any significant difference in NDVI and the application of Microgro Bioinoculant alone and in combination with Microlife Humic statistically increased NDVI values only at V10 (Table 6). Malik (2018) tested the seed inoculation of four biofertilizers applied independently and with two biofertilizers on barley (*Hordeum vulgare* L.) and none affected NDVI compared to the control (uninoculated). Other studies report no effect of biofertilizers on NDVI in wheat (Dal Cortivo et al. 2018) and soybean (Cerezini et al. 2016). Regarding this variability of biofertilizer performance on NDVI values, two wheat studies report that biofertilizer application did impact NDVI but other factors also influenced the magnitude of effects. Singh et al. (2019) assessed the integrated use of different biofertilizers and synthetic fertilizers and each biofertilizer impacted NDVI differently even under the treatments with the same synthetic input. Furthermore, the findings of Pagnani et al. (2019) showed significant differences in NDVI not only when the same biofertilizer was applied using different methods but also within three wheat varieties. Therefore, management aspects such as the nature of the biofertilizer, application method, crop, cultivar, interaction with other fertilizers and

environmental conditions can affect the overall performance of biofertilizer on NDVI values. This statement might be valid for other parameter such as PE, greenness, vigor and yield as well.

The effects of HA application on PE was not the same as NDVI. Generally, HA application affected PE values more frequently than NDVI considering the number of statistically significant results for both parameters (Table 6). According to Sellers (1987), NDVI can be an important index assessing biomass, leaf area index, absorbed photosynthetically active radiation and canopy photosynthetic capacity. However, this index might fail to assess some dynamic physiological mechanisms (Gamon, Peñuelas, and Field 1992) such as PE. The NDVI values of drought-tolerant evergreens were similar to what was observed in the current experiment, where NDVI did not proportionally change in accordance with variation in photosynthetic activity values (Running and Nemani 1988). Photosynthetic efficiency was greater with application of Microlife Humic than Monty's in 2018 and 2019 as well, based on frequency of statistically significant values in the individual and integrated application with biofertilizer. According to Haghighi, Kafi, and Fang (2012), even though the HA mechanisms to influence the biological activities in plants is not well defined, the application of HA on lettuce maximized photosynthetic activity due to enhancement of chlorophyll content and mesophyll conductance. More specifically regarding PE, using the same metrics F_v/F_m (maximum quantum yield of photosystem II), there are several studies reporting that HA can increase PE, especially when the plants are exposed to stress conditions. Ozfidan-Konakci et al. (2018) used wheat to test two doses of HA with and without stress imposed by endogenous cadmium. The application of HA only significantly inhibited the reduction of F_v/F_m on the plants under cadmium stress. Furthermore, application of HA increased values of F_v/F_m on blond plantain (*Plantago ovata*)

and mitigate the damage caused by salinity stress, possibly due improved nutrient uptake and physiological changes (Gholami, Samavat, and Ardebili 2013). Lotfi et al. (2018) tested the application of HA on rapeseed (*Brassica napus* L.) under different water regimes and found higher F_v/F_m values. The PE or maximum quantum yield of photosystem II measured by F_v/F_m is a very susceptible component of the photosynthetic process and according to Zhang and Sharkey (2009), stress conditions can result in an over-reduction of the electron transport chain impacting photosynthetic efficiency.

The significant effects of the biofertilizers on PE values were more frequent than with NDVI, similarly to what happened to HA application (Table 6). A study previously mentioned also found chlorophyll content values significantly higher with the application of biofertilizer (Cerezini et al. 2016). Even though, chlorophyll content and PE can be relatable to some extent, this is further evidence that biofertilizers influence plants differently when comparing NDVI and PE. Generally, the influence of biofertilizers on PE was variable when considering the number of statistically significant values. However, SoilSoup and Microgro Biostimulant showed slightly lower efficacy than Microgeo on PE values. Microgeo application did not result in more statistical differences in PE as it did for NDVI values when comparing with the other biofertilizer products (Table 6). Photosynthetic efficiency has been widely used as a tool to evaluate the physiological condition of plants, especially to diagnose stress levels (Lichtenthaler and Rinderle 1988, Mészáros et al. 2001, Tóth et al. 2002). A reduction in PE values might be caused by damage to photosystem II (PSII) which can promote a reduction in F_v/F_m (Porcar-Castell et al. 2014). Thus, PE values can be used to predict a plant's ability to mitigate stress. Gajdos et al. (2012) assessed the effects of a biofertilizer on corn and sunflower (*Helianthus*

annuus L.) with and without cadmium stress, and the biofertilizer mitigated stress via higher F_v/F_m only on corn. This again illustrates that the same biofertilizer can differentially affect PE values of different plant species even under the same environmental conditions. Another study addressing corn found that biofertilizer application mitigated the negative effects of nitrogen deprivation, where the treatments receiving biofertilizer had significantly higher F_v/F_m under lower rates of nitrogen (Veres et al. 2007). Biofertilizers derived from different sources have also positively influenced PE values of safflower (*Carthamus tinctorius* L.) (Raheleh, Ghassemi, and Asghari 2019)and aloe vera (*Aloe barbadensis* Miller) (Khajeeyan et al. 2019) under drought stress and banana without any promoted stress conditions (*Musa sapientum* AAA group 'Kluai Hom Thong') (Theerawitaya et al. 2017).

Vigor, and, to a greater extent greenness and vigor were affected by HA application (Table 6). Similarly to our results, two previous studies have shown that HA increased F_v/F_m values and visual greenness. Zhang et al. (2002) tested the effects of HA alone and in combination with seaweed and $FeSO_4$ on creeping bentgrass (*Agrostis palustris* Huds.) in two growing seasons. Application of HA alone or combined with other products resulted in greater F_v/F_m and greenness, but of differing magnitudes depending on the treatment and growing season. They also found that the increased F_v/F_m values did not necessarily correspond to greater greenness as we observed in our experiment in 2018 and 2019. Another study addressing the use of HA and seaweed on creeping bentgrass also reported greater F_v/F_m values and turf quality (visual score which greenness and vigor are considered) over two growing season (Zhang, Ervin, and Schmidt 2003). Evidence of the specific mechanism through which HA influences NDVI and PE is lacking. However, a review made by Canellas et al. (2015), provided a detailed

understanding of the potential HA pathways to impact nutrient uptake and plant metabolism. In summary, this review presented evidence of the biostimulant effect of HA on structural and physiological changes in roots and shoots related to nutrient use efficiency. Additionally, they describe the important influence on plant primary and secondary metabolism associated with mitigating stress conditions. Regarding secondary metabolites, greater levels of auxins (Aldesuquy 2000) and glycine-betaine (Papageorgiou, Fujimura, and Murata 1991) are related to cell membrane stabilization and consequently greater efficiency of photosystem II (PSII) when plants are under stress. Thus, these factors might elucidate the effects on NDVI, PE (maximum quantum yield of photosystem II), greenness and vigor (plant quality).

The application of HA resulted in similar ratings, likely due the fact that visual greenness and vigor estimates can be relatable, and these data were collected at the same time. However, estimates of greenness seemed to be slightly more affected by the application of biofertilizers compared to estimates of vigor (Table 6). Baltzoi et al. (2015) evaluated the effects of three biofertilizers on turfgrass (*Festuca arundinacea*) under 50% and 100% water regimes, and the application of two biofertilizers compensated for the lack of water on the treatments under 50% water supply, showing similar turfgrass quality to full irrigation treatments. Therefore, it shows the potential of biofertilizers increasing quality parameters as greenness and vigor under stress conditions.

3.3.1 Corn yield

Table 7. Effects of the biostimulants treatments on corn yield collected in Blacksburg, 2018 and 2019 and Champlain, 2018. ¥

Subcategory	Biostimulant treatment	Difference between treatments and control values			
		Yield, kg ha^{-1}		Yield, bu ac^{-1}	
Humic	Monty's	322.44		5.76	
	Microlife Humic	734.13		13.68	
Biofertilizer	Microgeo	1020.13		18.80	
	SoilSoup	120.75		1.86	
	Microgro Bioinoculant	557.30		10.63	
Humic + Biofertilizer	Monty's + Microgeo	1204.69		22.21	
	Monty's + SoilSoup	841.69		15.65	
	Monty's + Microgro B.	772.75		14.08	
	Microlife Humic + Microgeo	1713.81	*	30.25	*
	Microlife Humic + SoilSoup	1190.25		22.01	
	Microlife Humic + Microgro B.	1133.81		20.55	
	Actual Control Values	9123.13		178.42	

¥ The data from 2018 and 2019 were combined due the lack of significance (treatments x growth stages x years interaction).
* Means separation are significantly different as $\alpha<0.05$.

The integrated use of HA and biofertilizer (Microlife Humic + Microgeo) was the only treatment that resulted in corn yield greater than the control in 2018 and 2019 studies (Table 7). Even though only one treatment among twelve presented significantly higher grain yield, the results from 2018 and 2019 combined more closely approximated our initial hypothesis which was that synergy of HA + biofertilizer applied together would increase corn yield compared to each of the products applied individually and the control. Darvishi et al. (2010) reported strong correlation between corn PE (F_v/F_m) and corn grain yield, in contrast to what was observed in 2018-2019, where differences in PE were observed with various treatments only one regarding grain yield. In fact, only one treatment among six treatments under the integrated use of HA and biofertilizer increased corn yield, but an increment of 1714 kg ha^{-1} (18%) is substantial.

Furthermore, all the treatments showed numerically higher yield when comparing to control and the numbers had to be higher than 1205 kg ha^{-1} or 22 bushels $acre^{-1}$ in order to present significance in the statistical analysis. In a practical manner, the yield increment presented by all the treatments might be considerable to the producer but in scientific standards the level of significance was set and not reached.Canellas et al. (2013) conducted two experiments (greenhouse and field trials) to evaluate the effects of HA + biofertilizer on corn development. In the greenhouse experiment, the individual and combined application of HA and biofertilizer generally increased plant metabolism (plasma membrane H+-ATPase activity), sugar content and net photosynthesis. The treatment receiving the optimal dose of HA + biofertilizer had the greatest level of viable bacterial cells in root tissues. In the field trials, the combination of HA + biofertilizer increased corn yield in 65%. Application of HA increased corn root exudates into the rhizosphere (Puglisi et al. 2008, Canellas et al. 2008), thus the biofertilizer could be used as a carbon substrate by microbes. Another study addressing corn reported that application of HA, either alone or with biofertilizer maximized corn yield, however the highest gains were observed by the integrated use of both compounds (Baloach et al. 2014). The integrated use of HA and biofertilizers resulted in greatest wheat grain and straw yield, likely due the enhancement if enzymes activity, available nutrients in the rhizosphere and photosynthetic pigments (Abou-Aly and Mady 2009)

CONCLUSION

These studies were designed to better elucidate the effects of the individual and combined application of HA and biofertilizers on corn also receiving conventional fertilizing practices. The individual and combined application of HA and biofertilizer following the Integrated Plant

Nutrition System (IPNS) generally influenced corn development, to varying degrees. In 2017, corn height, NDVI, greenness and vigor were sensitive to the application of biostimulants in different magnitudes and growth stages, however grain yield and nutrient content were not affected. In combined studies from 2018 and 2019 corn height was not impacted by biostimulant application but NDVI, PE, greenness and vigor were affected at different doses and corn growth stages. The combined use of HA + biofertilizer (Microlife Humic + Microgeo) was the only treatment leading to increased grain yield. In sum, these studies provided evidence that the individual and combined application of HA and biofertilizer can influence corn growth and vigor at various points during the growing season. However, the current study cannot conclusively affirm that the integrated use of HA and biofertilizers (IPNS) is a better practice than the application of each compound individually. The intensive labor required to apply these biostimulants four times during the growing season is also a factor to be considered, but the objective of this study was strictly to assess the efficacy of the products on corn development. Therefore, further studies addressing different application frequency and rates should be conducted to validate these findings and contribute further to the understanding of the value and viability of the IPNS approach in a farming scene.

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