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The Synergistic Effects of Humic Substances and Biofertilizers on Plant Development and Microbial Activity: A Review

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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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. Some humic products are derived from lignin found in wheat straw and biofertilizers as compost and manure teas can be made using residues. Also, these biostimulants might decrease the necessity of synthetic inputs. This review strives to enhance our understanding of the conjunctive use of humic substances (HS) and biofertilizers. The biostimulant effects of each of these compounds are

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shown in the literature. Thus, our review question is whether the combined application of HS and biofertilizers can promote synergy between both compounds and potentially more efficacy. The effects promoted by using HS plus biofertilizers on plants and microorganisms are very interconnected, so sometimes these effects can be confounded. For instance, the root elongation promoted by HS might increase hyphal fungi colonization. Therefore, this review as divided in three sections: Responses of plants, fungi and bacteria. The findings indicate that the source and application rate of HS will have a strong impact on whether plant growth and microbial activity significantly improved. The microbial species and plant type also influence the response to HS. The prospects of the conjunctive use of and biofertilizers to stimulate plant development and microbial activity in agricultural systems are theoretically substantial when considering the total number of studies included in this review.

Keywords: Biostimulants; humic acid; humate; plant growth; compost tea; manure tea.

1. 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 and decrease the need of synthetic inputs.

The current financial 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 negative 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 biological 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 substances (HS) and biofertilizers as an organic and biological resource, respectively.

Humic substances are generally classified into humic acid (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 HS are not naturally occurring in soil. They stated that minerals and enzymes involved in the HS synthesis are not relevant in natural systems when observed by modern analytic techniques. However, in both scenarios the use of humic products in agriculture could promote positive effects in a soil containing or not preexisting HS 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 HS 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 HS 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, HS 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 HS + 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 HS plus biofertilizers positively affected yield but also presented the highest values when comparing to the alone application and/or control (no HS and biofertilizer). Also, they described the individual biostimulant effect as the main mechanism of action for HS and biofertilizers. Thus, the individual impact of HS and biofertilizers on primary and secondary metabolites plus nutrient uptake [43] can have important consequences on yield. Regarding the complementary effects of HS on biofertilizers and vice versa, HS stimulated microbial activity through ion exchange and metal complexing (chelating) systems [44,45] and taking into account that HS 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 HS 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 HS and the bioinoculant (Herbaspirillum seropedicae) were applied in conjunction, compared with the individual use of HS and bioinoculant, respectively. Interestingly, the encapsulation of biofertilizer living cells in alginate beads enriched with HS enhanced shoot and root length on tomato and effectively protected the biofertilizer from the adverse condition of the soil [49]. Our review question is whether the combined application of HS and biofertilizers can promote synergy between both compounds and potentially more efficacy. Thus, the objective of this review is to have a better understanding of the effects of HS 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 HS on plants, fungi and bacteria.

2. MATERIALS AND METHODS

To assess the effects of the conjunctive use of HS 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 HS + biofertilizers. The variability of these articles in terms of source and dose of HS, 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 HS + biofertilizer. Furthermore, to promote a fair equivalence of values to compare very different experimental conditions, we used the increment and/or decrement of each parameter in percent (%) units when comparing to the control values. Control was defined as the treatment not receiving HS, biofertilizer or both, in order to clarify the understanding of each category. In fact, we recognize that the effects promoted by HS and biofertilizers on plants, fungi and bacteria are very interconnected, even taking actions to differentiate each category. Thus, these effects can be confounded. For instance, the root elongation promoted by HS could also increase the microbial colonization in the roots [52,53].

3. RESULTS AND DISCUSSION

3.1 The Effects of Humic Substances and Biofertilizers on Plants

Generally, the fourteen studies presented in Table 1 evaluate plant effects when HS and biofertilizer are applied in combination and when each compound is applied independently. Each study has its own specificities related to HS 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) HS, 3) biofertilizer and 4) HS + 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 HS + 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 HS 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 HS + 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 HS + biofertilizers for all the cases. Therefore, these values might reveal that the benefits of the combined use of HS + biofertilizers can be expressed in different magnitudes. Moreover, different plants and environments will affect the performance of each treatment. 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, HS 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 HS might potentially improve plant productivity.

On the other hand, among the studies presenting mixed (positive and negative) 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 were not consistent in terms of one specific treatment showing only positive or negative results. In these cases, the treatments containing HS, biofertilizer and the combination of HS + biofertilizer presented 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. Overall, the application of HS + biofertilizer showed to be positive only in certain parts of the plants and in specific rates. For instance, Study 3 [58] showed that the HS 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 HS rates increased. The highest rate of HS 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 HS. Therefore, inappropriate HS rates might harm the positive effects of the conjunctive use of these compounds. In other words, rates extremely low or high can simply be ineffective or to prejudice plant development.

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	155 gh		17.3	

megaterium cells				
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 HS, biofertilizer and humic + biofertilizer treatments promoting positive and negative effects, made it not very clear in terms of how beneficial the application of these compounds is. Therefore, we compiled the entire Table 1 in Fig. 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 HS + biofertilizer were present in the whole list of studies. The Fig. 1 confirmed the synergy of the integrated practice, where HS + biofertilizers presented the highest value on the stimulation of plant development (Fig. 1). We averaged the values of each treatment for every study presented in Table 1 and compared to the control treatment. The results showed that HS, biofertilizer and HS + biofertilizers treatments presented 11%, 16% and 29% higher effects on plants, respectively (Fig. 1). Indeed, Fig. 1 is combining many different plant species, growth and yield parameters, sources, and rates of HS and biofertilizers. However, we had to unify this information because there are not many studies specifically addressing the combined use of HS + biofertilizers in similar plant species and environment. Then, it can be a broad guidance for the general use of these compounds. The independent use of HS 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, Fig. 1 may represent one or more effects of these mechanisms caused by the application of HS, biofertilizers and/or both.

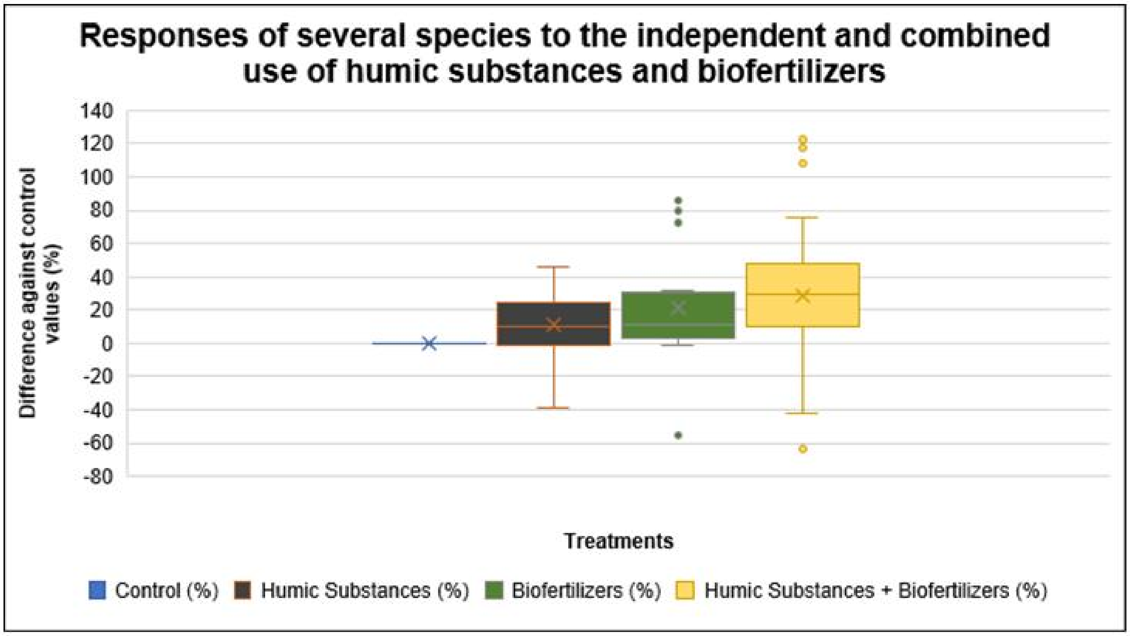

Fig 1. The compilation of results of 14 studies presenting the effects of the independent and integrated use of humic substances and biofertilizers on several plant species under different growing conditions

3.2 The Effects of Humic Substances and Biofertilizers on Fungi

The responses of fungal growth under increasing rates of HS 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 HS 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 HS + biofertilizer compared with the control (only biofertilizer, no HS). The biodegradation of HS by fungi has been showed in the literature [70,71]. Thus, a possible explanation for the mycorrhizal growth increment by HS 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 HS rate under pH 4 resulted in 80% higher dry weight of Ectomycorrhizal fungi when comparing to control, but the greater dose of HS decreased it 37%. Also, in the same study Ectomycorrhizal fungus under pH 7 were not responsive to the application of 3200 ppm of HS (Table 2). Again, the HS doses appearing as an important factor on the performance of these compounds. Interestingly, Zhou and Banks [72] affirmed that the HS 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 HS are harming fungal development. Differently than

Study 15 and 16, it showed only negative effects on fungi growth. The control (no HS) presented the highest value of hyphal length and as the HS 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 HS rate (3200 ppm) resulted in the lowest values of Ectomycorrhizal fungi. However, in Study 15 lower rates of HS enhanced ectomycorrhizal growth and it did not occur in Study 3. These studies show the extreme importance of HS 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 HS rates, environments, plants and microorganisms might cause variable results.

The HS rates of studies 15, 16 and 3 were converted to the same unit (parts per million) and compared on Fig. 2. Clearly, HS 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 HS.

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

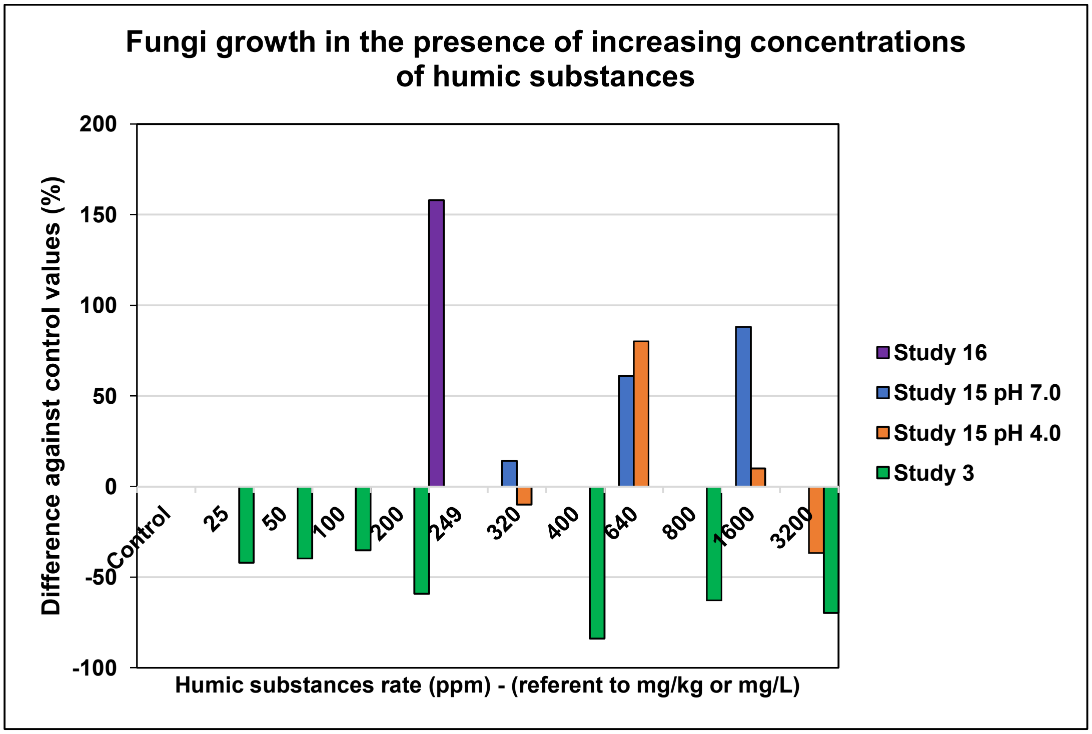

Fig. 2. The responses of mycorrhizal growth to increasing application rates of humic substances presented in Study 3, 15 and16

3.3 The Effects of Humic Substances and Biofertilizers on Bacteria

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 HS. 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 HS 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 HS contributed to bacteria development (Table 3). The three bacteria strains tested in Study 12 responded positively to the application of HS, where none of them were incapable to grown under HS. Study 17 showed 108 soil and earthworm bacteria strains capable on growing under HS application and 55 being incapable. Study 2 and 18 presented 2 bacteria strains (one each) where HS showed to be beneficial for bacterial growth. Finally, Study 19 did not specify the exact number of strains, but several microorganisms enhanced growth after HS 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 fluorescens*, *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 HS 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 HS; then it might reinforce the hypothesis that HS 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); microorganisms 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	Not applicable		
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			

Table 4. Effects of humic substances 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 microorganisms (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 and 19 did not provided statistical information for these parameters.*

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

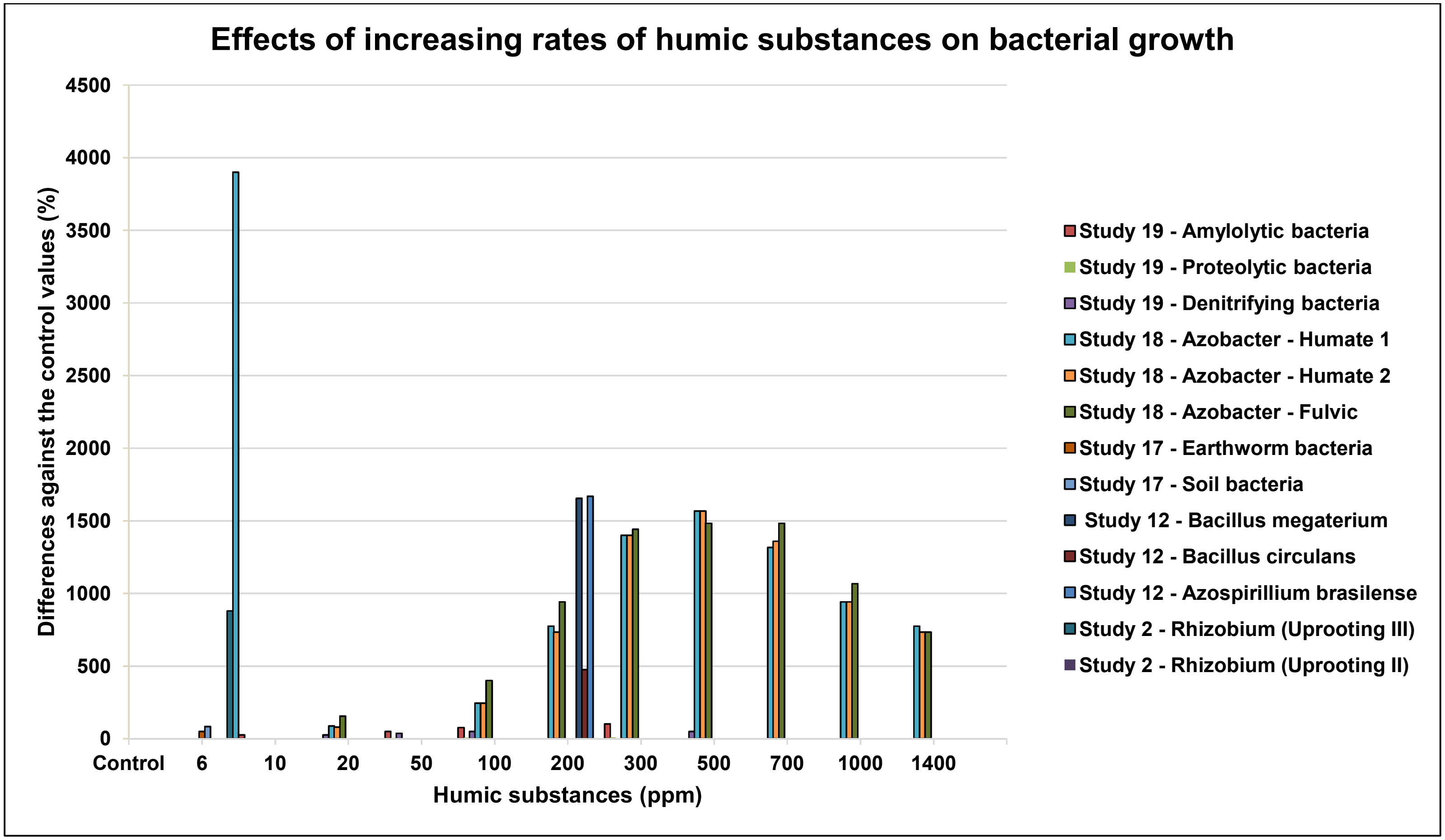

Fig. 3. Responses of bacterial growth under increasing rates of humic substances. The parameters used to measure the bacterial growth were number of nodules (Study 2), most probable number (Study 12), maximal specific growth rate (Study 17), number of cells (Study18) and number of microorganisms (Study 19)

The HS concentration rate was not considered in Table 3, therefore we simply assumed and counted the growth of bacteria when any rate of HS was added. Furthermore, Table 3 was basically enumerating the bacteria strains that can grow in conditions where HS are added; it is not a comparison to identify if the use of HS is better than to neglect its use. However, the studies cited in Table 3 provided information regarding the application or absence of HS, which was discussed in Table 4. There, the application rate was considered in order to conduct the comparison between the use or not of HS on bacterial development. In fact, the HS concentration or rate is extremely important as this review has shown previously in the plant and fungi interaction (Figs. 2 and 3). It was not different when addressing the bacteria interaction with HS. Thereby, Table 4 included the studies mentioned in Table 3, but the HS concentrations were assessed, and their effects compared. The optimal application of HS 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 HS rate. In other words, the bacterial growth was not directly proportional to the increment of HS concentration. In fact, we compared different bacterial strains and many methods to measure bacterial growth. Therefore, the magnitude of increase from one study to another is very high. Although, generally the application of HS increased microbial activity, where some rates promoted higher effects.

The HS application rates from study 2, 12, 17, 18 and 19 were converted to the same unit (parts per million) and compared in Fig. 3. The studies evaluating only one rate of HS (Study 2, 12 and 17) showed that the application of HS increased bacterial growth when comparing to control where no HS were used. The studies testing more than one HS rates (Study 18 and 19) generally showed that bacterial biomass started to increase when the first doses of HS were applied, then the biomass reached the maximum value or plateau. Posteriorly, the HS concentration continued increasing but the bacterial biomass started to drop consistently. Again, it represented the importance of an appropriate application rate when HS 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.

4. CONCLUSION

This review has shown that the response of plant development and microbial activity to HS, 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 HS and biofertilizers will have a strong impact on whether plant growth and microbial activity will significantly improve. The plant and microbial species also influence the response to HS. Furthermore, the interactions between each of these different factors in the presence of HS 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 HS + plants + microorganisms 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 HS and biofertilizers to stimulate plant development and microbial activity in agricultural systems are theoretically substantial. However, the number of publications is scarce when considering similar plant species, environment, HS source and microorganisms contained in the biofertilizer, then more research is necessary.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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