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Comparative Evaluation of Humic Substances: Effect at Cell Level and Chlorophyll Retention during Accelerated Senescence

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Abstract

The influence of humic substances (HS) formulations derived from sedimentary and compost sources was studied on plant growth at cell level and chlorophyll retention during accelerated senescence of leaf tissue. The direct effect of HS formulations was studied on cell expansion using cucumber and radish cotyledon expansion test. The cucumber hypocotyl elongation test was used to study the effect on cell elongation. Chlorophyll pigment retention in excised leaf tissue incubated in dark with high temperature was assessed to study the effect on leaf senescence. Explant tissues were incubated directly in the solutions of the formulations at the concentration recommended for foliar application to the crop plants. HS formulations showed significant variations in their direct bio-stimulatory effects. Formulations derived from compost sources were found superior in terms of inducing a direct stimulatory effect on cell expansion and cell elongation and in maintaining chlorophyll pigment retention during accelerated senescence. HS from sedimentary sources stimulated cell expansion and delayed chlorophyll degradation to a lesser extent compared to HS from compost. However, HS formulations derived from sedimentary sources used in this study were not effective in inducing cell elongation in the cucumber hypocotyl elongation test. The direct bio-stimulatory effect of HS formulations differed significantly between the formulations that were evaluated.

Keywords

Biostimulants, Chlorophyll, Compost, Cotyledon, Humic Substances, Hypocotyl, Sedimentary

1. Introduction

The application of humic substances (HS) has been shown to improve crop growth and productivity in many crop species under diverse agroclimatic conditions [1]. However, the mechanisms responsible for the increased productivity induced by HS are being debated. Two hypotheses have been proposed to explain the mechanisms responsible for enhanced crop growth and productivity. These hypotheses were broadly divided into indirect and direct influence hypotheses. The indirect influence hypothesis explains that HS improves nutrient availability in the soil and increases nutrient uptake by plants through various means, such as chelating nutrients in the soil and facilitating root uptake, enhancing soil structure, improving water holding capacity, increasing Cation Exchange Capacity, reducing nutrient leaching, and enhancing soil microbial activity [2]-[7]. All these improvements were claimed to help the crop plants to absorb more nutrients and water resulting in enhanced growth and productivity.

The direct influence hypothesis explains that HS directly triggers plant growth and developmental processes by affecting the transcriptional and post-translational regulations of several enzymes and molecular transporters in plants by acting similarly to many endogenous plant growth hormones [8]. HS were shown to mimic growth stimulations similar to auxins, gibberellins, cytokinin and other hormones [9]-[14]. Humic substances were considered similar to phytohormones to manifest the effect in plant cells through gene activation at transcriptional and post-translational modifications of signalling entities that trigger different molecular, biochemical and physiological processes [15] [16]. Plant growth stimulation at the cell level involves an increase in cell division, cell elongation, cell expansion and morphogenesis. The involvement of growth-stimulating substances at the cellular level can be demonstrated through established bioassay systems standardized for phytohormones.

HS formulations available in the market for agricultural use are expected to possess both a direct and indirect influence on crop plants resulting in stimulated growth, development and crop productivity. [17] showed that the chemical structure and biological activity of humic substances define their role as plant growth promoters. The extent of stimulated crop growth response to HS however depends mainly on the source from which formulation is made, the rate of HS applied to the crop, and a lesser extent on the plant type and growing conditions [1]. HS formulations developed for agricultural use are generally made from two source materials either from sedimentary sources extracted from coal, leonardite and peat, etc. or extracted from partially modified organic matter like compost, farm yard manure, vermicompost and biologically modified vegetable biomass.

Compost and soil-derived HS have been shown to exhibit greater growth enhancement effects compared to HS derived from brown coal and peat [1]. [18] found that humic acid produced from composted material was efficient in increasing plant agronomic and physiological activity. Such an effect is likely to be related to the chemical structure and possibly also related to the extracted mineral nutrients in the formulations. [19] mentioned that HS particularly of low molecular mass are readily taken up by plants and promote plant growth and development. HS derived from freshly decomposing materials such as compost were usually expected to contain lower molecular weight distribution of molecules, aggregate, which has also been implicated in plant growth responses [1]. Low molecular weight molecules can enter through the plant cell membranes and stimulate plant metabolism resulting in enhanced growth at cell, tissue and organ level. In this study, the direct influence of HS formulations from sedimentary and compost sources was compared on plant growth at the cellular level, as well as the retardation of chlorophyll pigment degradation in leaf discs under simulated stress conditions..

2. Materials and Methods

The relative direct bio-stimulatory effect of HS formulations extracted from sedimentary and compost sources was investigated. Ten randomly selected HS formulations from the shelf of Agrochemical outlets from Indian markets were selected. Five of these formulations viz., A, B, C, D & E were extracted from sedimentary sources (label claim on the bottle) and five others viz., F, G, H, I and J were from compost sources. The direct bio-stimulatory influence of the formulations was studied on cell expansion, cell elongation and on chlorophyll pigment retention during stimulated senescence of excised leaf discs. In all the experiments, the tissue incubation medium contained only the recommended dose of HS formulations diluted in distilled water without any other added supplements. The variations in growth or chlorophyll content in response to test solutions were compared with growth or chlorophyll content in the tissue incubated over distilled water alone, which served as control.

2.1. Influence of HS Formulations on Cell Expansion Growth of Cucumber Cotyledons

Seeds of cucumber, *Cucumis sativus* L. (cv Guntur Local) were surface sterilised using 1% sodium hypochlorite solution, rinsed well and soaked in sterile distilled water for four hours. Uniform sized seeds were germinated in petridishes lined with moistened filter paper in an incubator in the dark, and maintained at a temperature of 25˚C ± 2˚C for 72 hours. Germinated seed with a radicle length of 20 mm were selected. Seed coat and radicle was removed and cotyledons were separated. Twelve cotyledons were used per petridish (100 mm diameter) and four petridishes were used per treatment. Cotyledons were incubated with 6 ml test solutions in a growth chamber maintained at a temperature of 25˚C ± 2˚C, RH 80% and at a continuous light intensity of 1000 lux for 96 hours. At the end of incubation period fresh weight of ten uniform sized cotyledons were recorded, after surface drying the cotyledons using a blotting paper.

2.2. Influence of HS Formulations on Cell Expansion Growth of Radish Cotyledons

Seeds of radish *Raphanus sativus* L. (var. Pusa Chetki long) were used for the study. Similar procedures as used for raising cucumber cotyledons were used, however, out of the two cotyledons of the seed only the big cotyledon was used for the study and the small cotyledon was discarded.

2.3. Influence of HS Formulations on Cucumber Hypocotyl Elongation Growth

Surface sterilized seeds of cucumber *Cucumis sativus* L. (var. Guntur Local) were germinated in darkness for 72 hours at a temperature of 25˚C ± 2˚C in petridishes over filter paper media moistened with sterile distilled water. Germinated seeds with a seedling length of 10 mm hypocotyl were selected. Ten mm apical hypocotyl segments along with cotyledons were cut using sharp blade. Twelve uniformly thick hypocotyl segments were incubated in petridishes (140 mm diameter) with 10 ml of test solutions for 72 hours at a temperature of 25˚C ± 2˚C. Four replications were maintained per treatment. At the end of incubation period length of ten uniform sized elongated hypocotyls were measured using a scale.

2.4. Influence of HS Formulations on Chlorophyll Pigment Retention in Leaf Discs under Stimulated Stress Condition

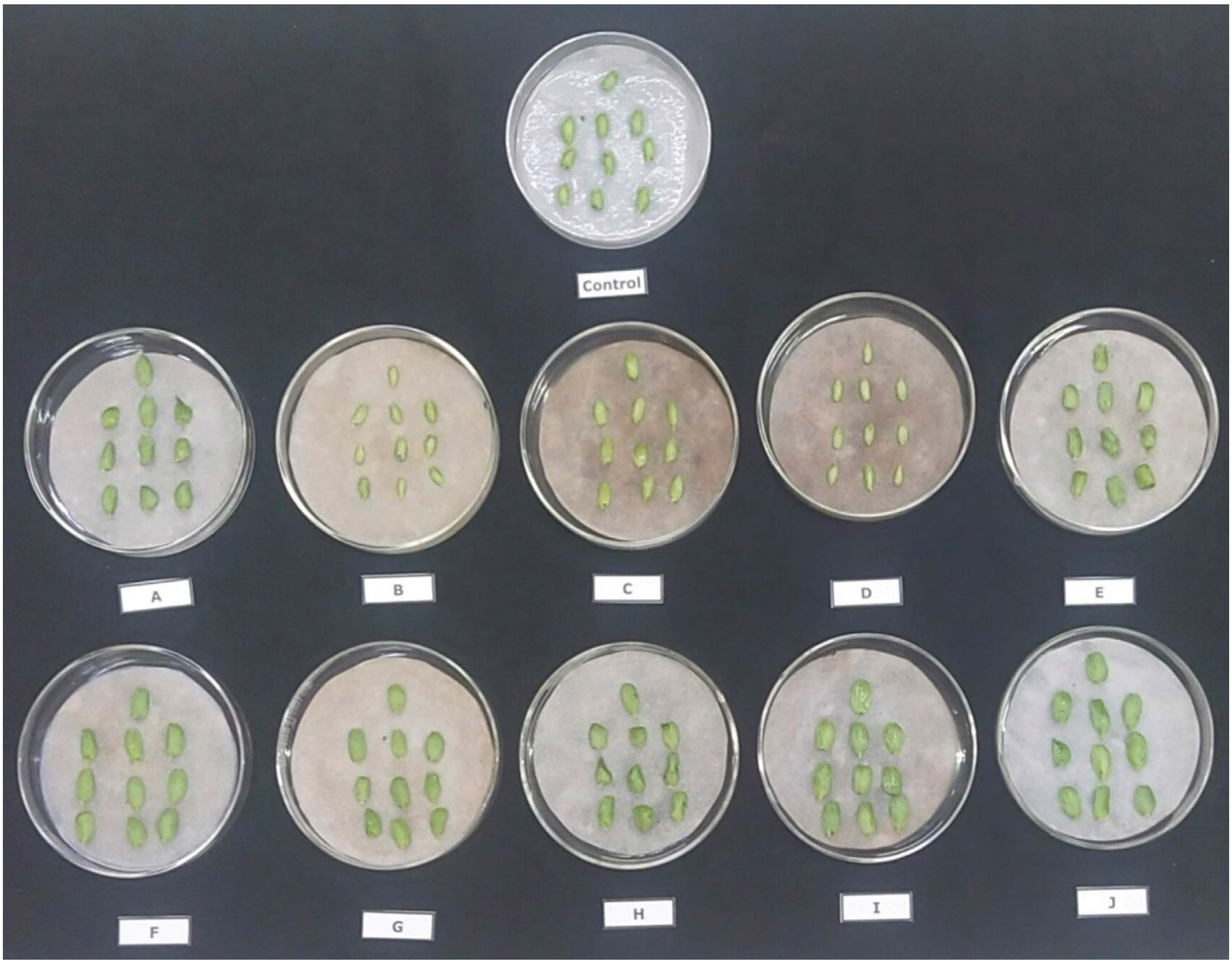
Fully expanded green leaves from the bottom canopy of field grown cowpea plants *Vigna unguiculata* (var. C-152) were selected for the study. Leaf discs of 6 mm diameter were made using a cork borer and 10 leaf discs were floated over 6 ml of test solutions taken in petridishes (60 mm). Four replicates were used per treatment. Leaf discs were incubated in dark at a temperature of 35˚C for 96 hours. At the end of 96 hours total chlorophyll content in the leaf discs were determined following the method by [20].

3. Results

3.1. Influence of HS Formulations on Expansion Growth of Cucumber Cotyledons

HS formulations from compost and sedimentary sources were effective in stimulating expansion growth of cucumber cotyledons. However, marked variations in expansion growth of cucumber cotyledons were noticed in response to different HS formulations (**Figure 1(A)**). HS formulations from compost sources in general resulted in more expansion growth of cotyledons, compared to HS from sedimentary sources (**Table 1**).

Percent increase in fresh weight of cotyledons in response to treatment with various HS formulations in comparison to cotyledons incubated in water is presented in (**Figure 1(B)**). All the HS formulations from compost sources induced more growth of cotyledons compared to HS formulations from sedimentary sources. Four out of five formulations from compost sources showed more than 50% increased growth over control. Three formulations (A, C, and E) from sedimentary sources showed less than 30% increase in growth and two formulations (B and D) inhibited cotyledon growth by 6.3% and 12.4% respectively.



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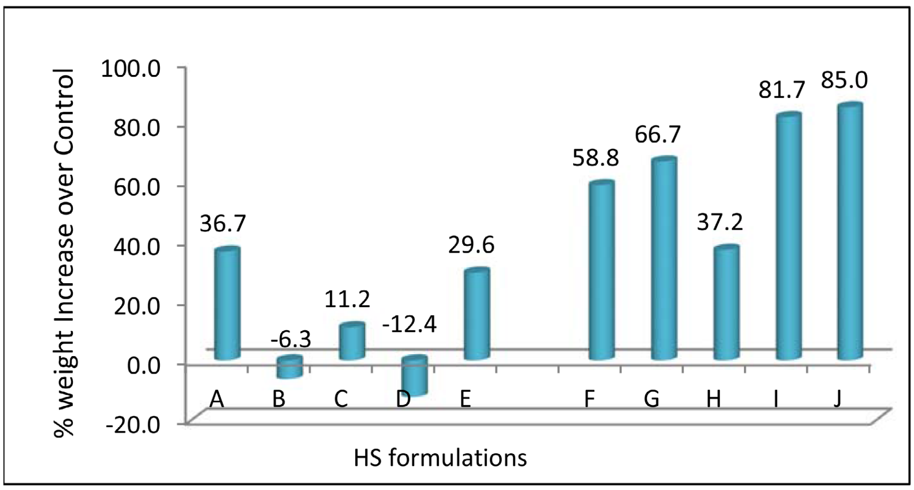

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Figure 1. (A) Cucumber cotyledons at the end of 96 hours incubation period in HS test solutions. Control (water) A, B, C, D & E are HS from sedimentary sources and F, G, H, I & J are HS from compost sources; (B) % increase of fresh weight of cucumber cotyledons in HS test solutions over control (water) after 96 hours of incubation period. A, B, C, D & E are HS from sedimentary sources and F, G, H, I & J are HS from compost sources.

Table 1. Fresh weight of cucumber and radish cotyledons, cucumber hypocotyl length and total chlorophyll content in HS test solutions. Control (water), A, B, C, D & E are humic substances from sedimentary sources and F, G, H, I & J are humic substances from compost sources.

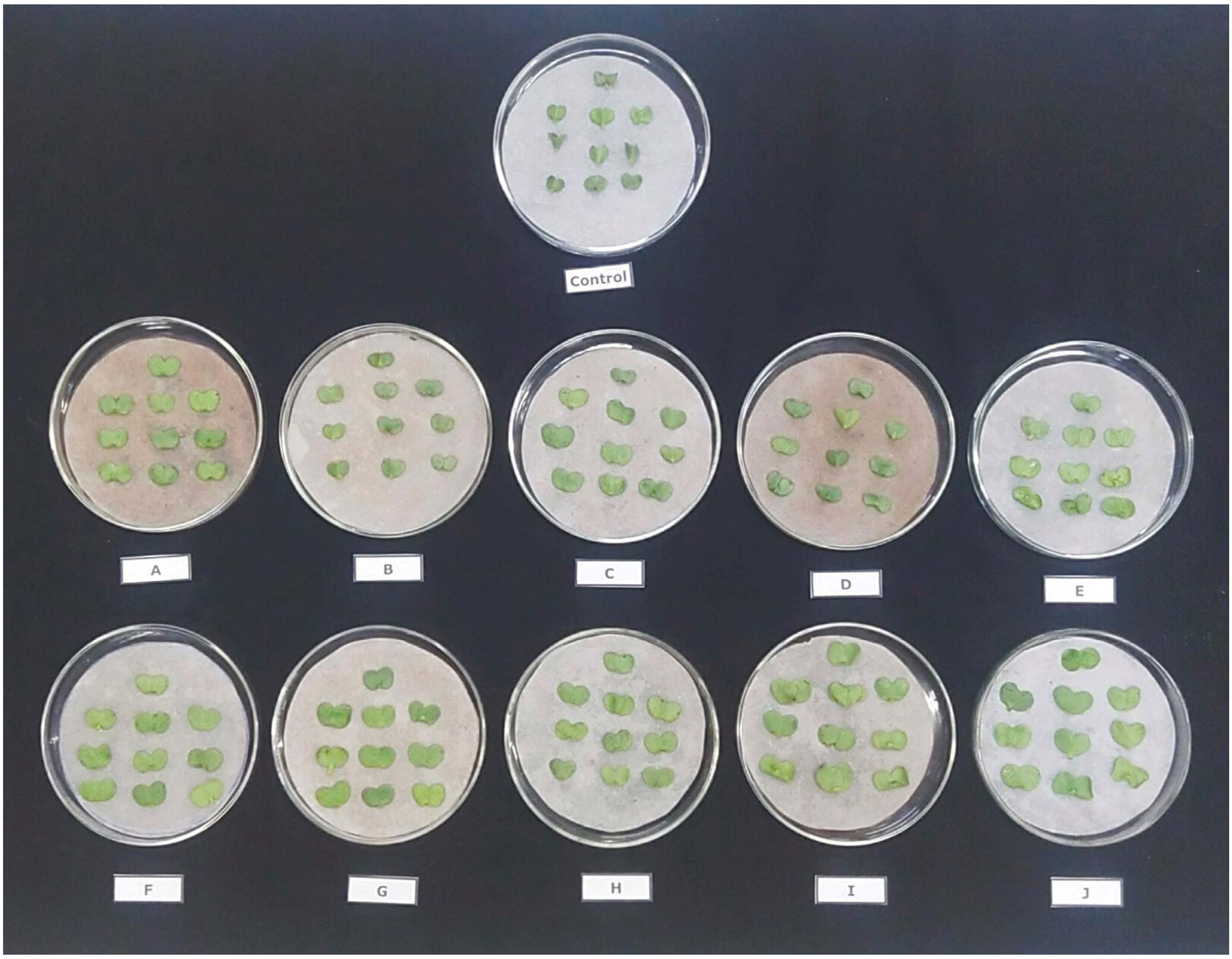
Treatments	Cucumber Cotyledon Fresh Weight (mg/10 cotyledons)	Radish Cotyledon Fresh Weight (mg/10 cotyledons)	Cucumber Hypocotyl length (mm)	Total Chlorophyll Content ($mg \cdot g^{-1}$ leaf fresh weight)
Control	178.6	335.9	34.2	0.38
HS Formulations from Sedimentary Source				
A	244.1	415.3	23.6	1.41
B	167.3	303.0	21.2	1.21
C	198.3	369.9	25.4	1.70
D	156.5	317.8	24.2	0.93
E	230.6	384.6	24.4	1.36
HS Formulations from Compost Source				
F	283.6	494.6	36.6	1.96
G	297.8	496.7	39.8	2.31
H	245.2	430.8	30.6	1.88
I	324.5	560.4	35.0	2.44
J	330.4	593.9	55.4	2.93
CV	**5.92**	**1.79**	**6.76**	**9.59**
CD@5%	**24.21**	**13**	**2.74**	**0.27**

3.2. Influence of HS Formulations on Cell Expansion Growth of Radish Cotyledons

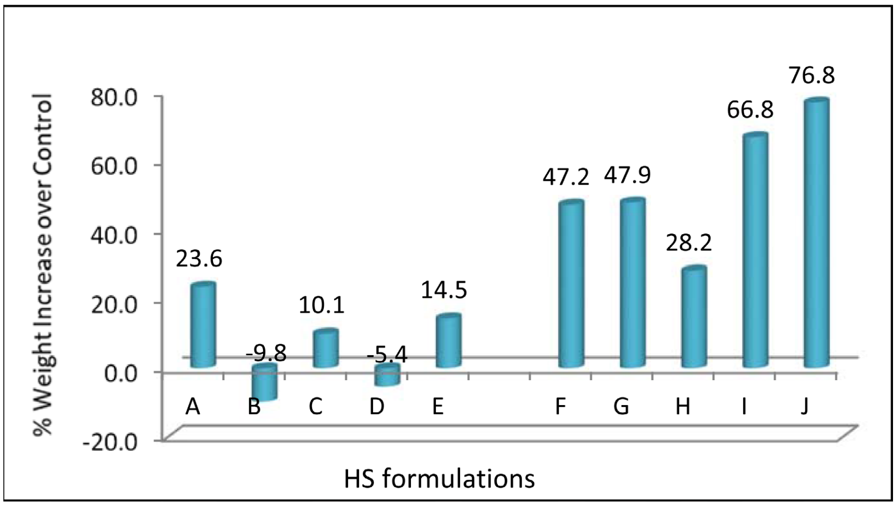
Significant variation in radish cotyledons growth was observed in response to HS formulations (Table 1, Figure 2(A)). All HS formulations increased cotyledons growth compared to growth on water (control). The growth response of radish cotyledons to different HS formulations showed almost similar trend as that of cucumber cotyledons. HS formulations from compost sources induced more growth stimulation as compared to HS formulations from sedimentary sources. HS from compost resulted in 28% to 76% increase in fresh weight (Figure 2(B)). Three formulations A, C, E from sedimentary sources resulted in 10% to 23% increase in weight. Two formulations B and D inhibited growth to an extent of 9.8% and 5.4% respectively.

3.3. Influence of HS Formulations on Elongation Growth of Cucumber Hypocotyl

Elongation growth of cucumber hypocotyl segments also showed significant



(A)



(B)

Figure 2. (A) Radish cotyledons at the end of 96 hours incubation period in HS test solutions. Control (water) A, B, C, D & E are HS from sedimentary sources and F, G, H, I & J are HS from compost sources; (B) % increase of fresh weight of radish cotyledons in HS test solutions over control (water) after 96 hours of incubation period. A, B, C, D & E are HS from sedimentary sources and F, G, H, I & J are HS from compost sources.

variations in response to HS formulations (**Table 1**, **Figure 3(A)**). The percent variations in final length of hypocotyl segments in relation to final length of segments incubated in water medium (control) is given in (**Figure 3(B)**). Four HS formulations from compost sources resulted in 2.3% to 62.0% increased growth of hypocotyl over water (control) and one formulation inhibited elongation growth by 10.5%. All the HS formulations from sedimentary sources inhibited elongation growth of hypocotyl segments. The extent of inhibition ranged from 25.7% to 38%.

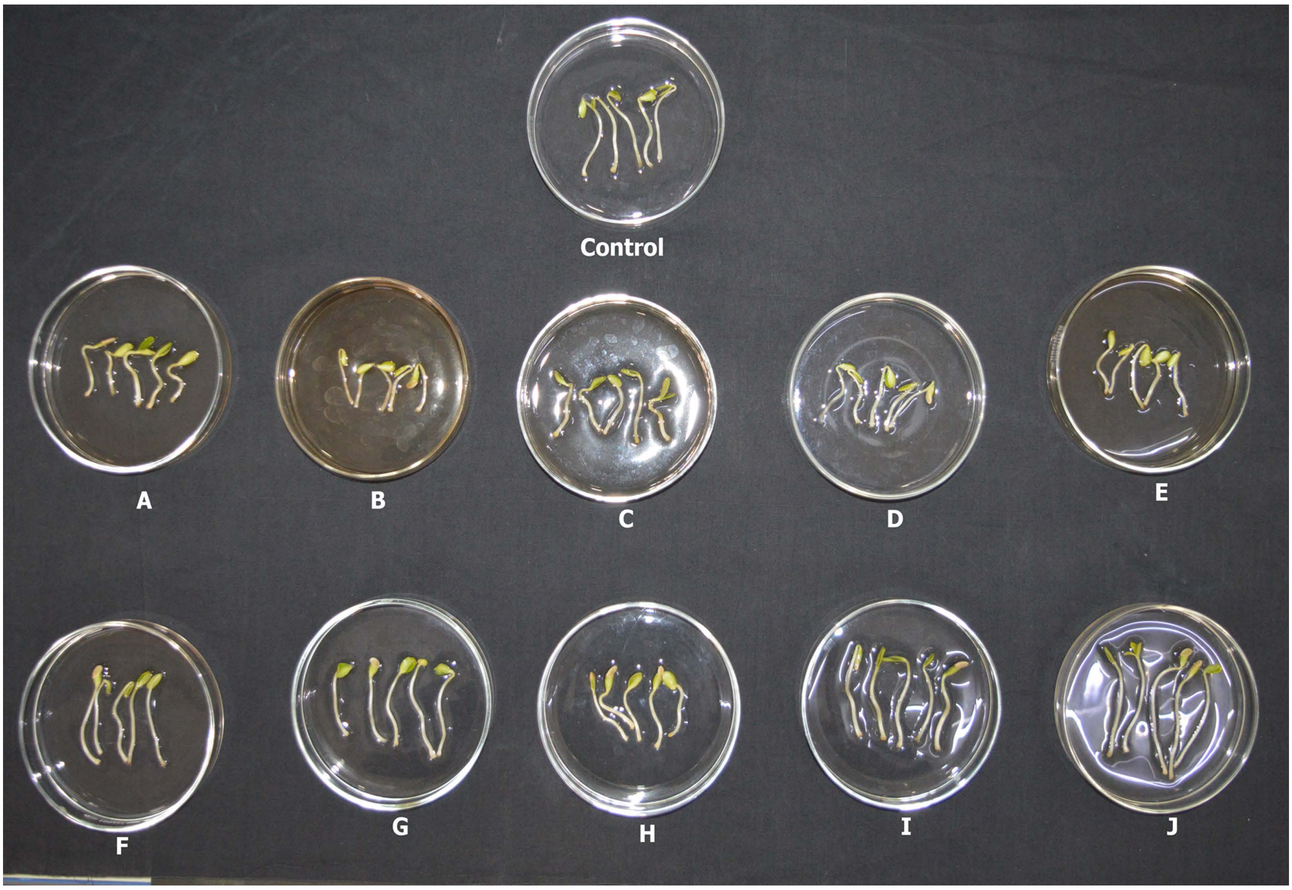
3.4. Influence of HS Formulations on Chlorophyll Pigment Degradation in Excised Leaf Discs under Stimulated Stress Conditions

All HS formulations were effective in delaying senescence of excised leaf discs under stimulated stress conditions as shown by maintenance of more chlorophyll pigment content (**Table 1**, **Figure 4(A)**) at the end of stress treatment compared to control. Leaf discs floated over HS formulations from compost sources retained more chlorophyll pigment content compared to HS formulations from sedimentary sources (**Figure 4(B)**). The chlorophyll pigment retained in leaf discs at the end of stress period in HS formulation from compost sources ranged from 1.88 to 2.93 $mg \cdot g^{-1}$ of leaf fresh weight, when compared to control. However, HS formulations from sedimentary sources showed only 0.93 to 1.7 $mg \cdot g^{-1}$ of chlorophyll pigment content in leaf fresh weight over control.

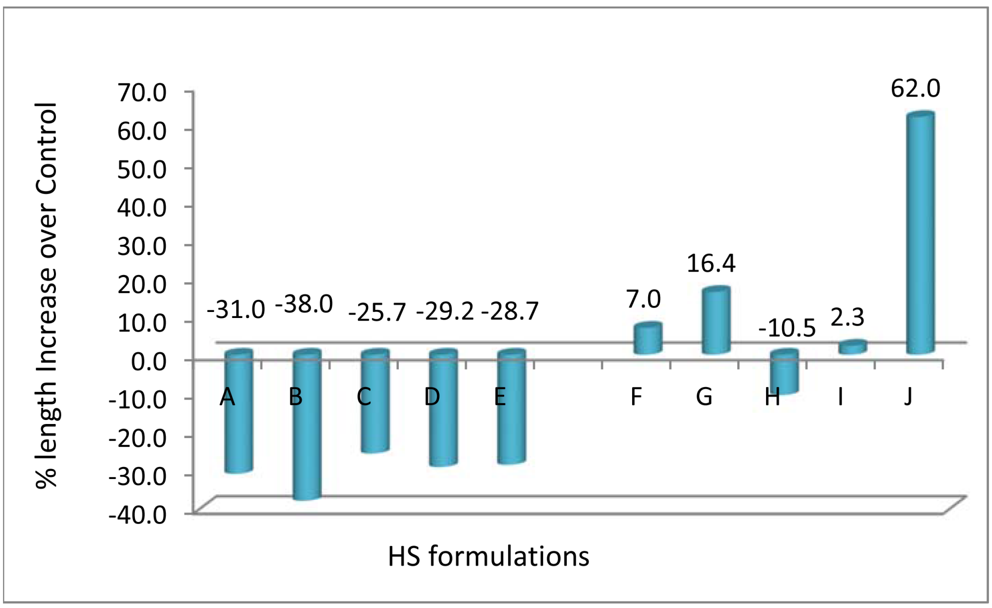
4. Discussions

The plant growth-stimulating influence of HS is associated with its direct effect at the molecular and physiological levels, resembling HS-like and phytohormone-like effects. Additionally, HS exhibits multiple indirect effects at the soil level, resulting in increased nutrient and water uptake by plants. The relative direct and indirect influence of HS on plant growth is associated with the source material from which HS are derived. A meta-analysis of published results on plant growth response of HS by [1] emphasised the importance of further studies to understand how a particular HS improves plant growth. They opined that such knowledge will help to open up door for tailoring HS formulations for specific responses. In the present investigation the differential influence of HS derived from sedimentary and compost sources on direct effect on cell expansion, cell elongation and chlorophyll pigment retention in leaf discs exposed to stimulated stress condition were studied.

The influence of HS formulations on growth at the cellular level was examined using two well-defined bioassays, namely cucumber and radish cotyledon expansion tests. Young cotyledon tissues also known as the first leaves were directly exposed to recommended concentrations of HS formulations (as per instructions on the label recommended for foliar applications), no other nutrients or cofactors are used in the growth media to influence growth. HS derived from compost sources stimulated growth of cotyledons in both cucumber and radish (**Table 1**).

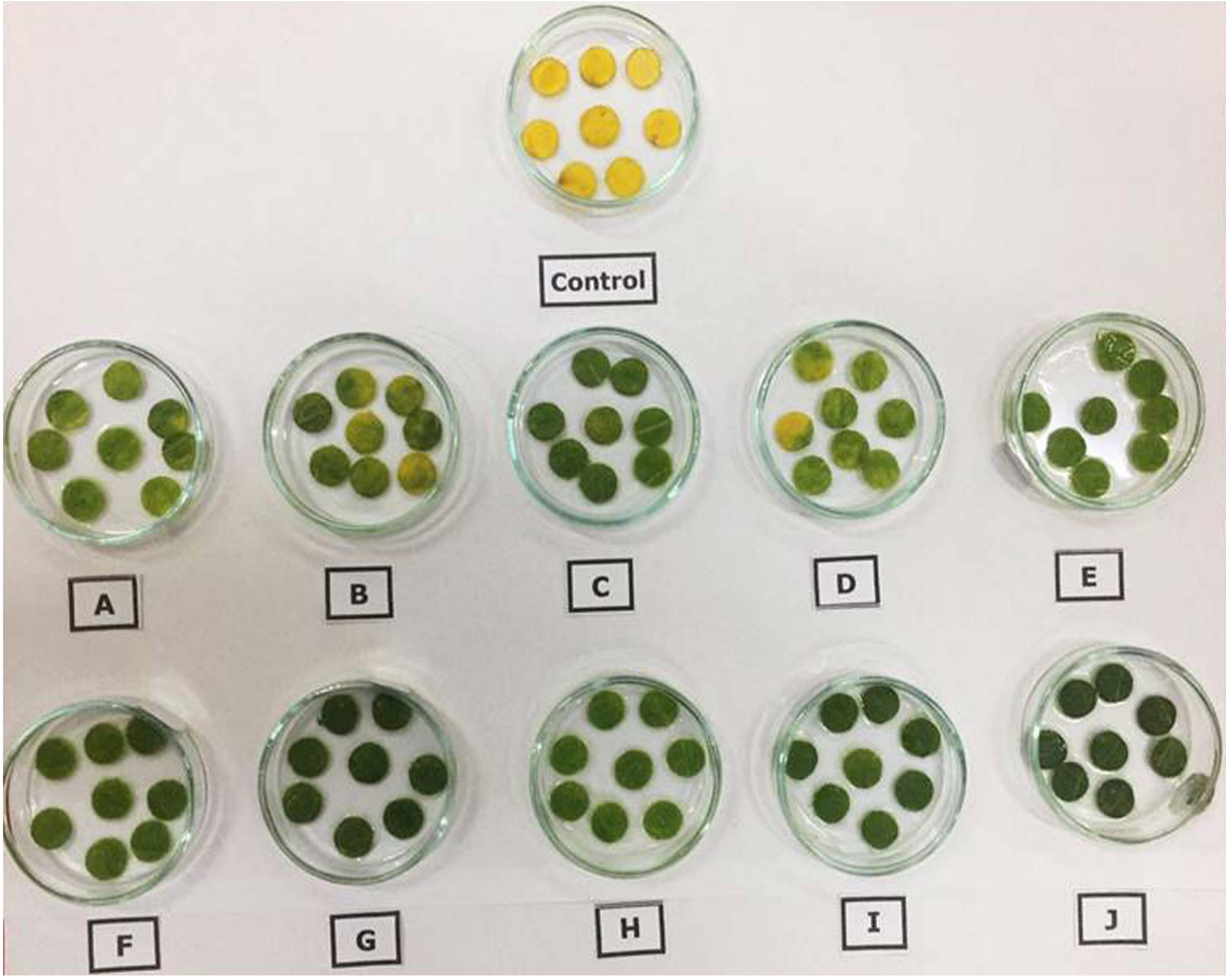


(A)



(B)

Figure 3. (A) Cucumber hypocotyl length at the end of 48 hours incubation period in HS test solutions. Control (water) A, B, C, D & E are HS from sedimentary sources and F, G, H, I & J are HS from compost sources; (B) % increase in cucumber hypocotyl length in HS test solutions over control (water) after 48 hours of incubation period. A, B, C, D & E are HS from sedimentary sources and F, G, H, I & J are HS from compost sources.



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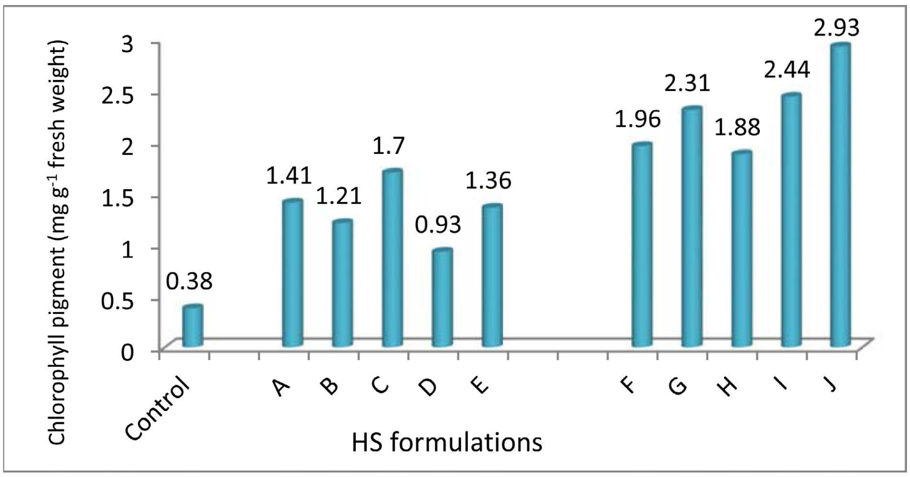

(B)

Figure 4. (A) Variations in chlorophyll pigments in cowpea leaf discs floated over control (water) or on test solutions for 96 hour. A, B, C, D & E are HS from sedimentary sources and F, G, H, I & J are HS from compost sources; (B) Total chlorophyll pigment (mg/g fresh weight) in cowpea leaf discs at the end of 96 hours incubation period on control (water) and HS test solutions. A, B, C, D & E are HS from sedimentary sources and F, G, H, I & J are HS from compost sources.

However, the extent of growth stimulation varied between the formulations. Formulation J and I are superior in their effect. Three of the HS formulations derived from sedimentary sources showed stimulatory effect and two formulations showed inhibitory effect on cotyledon growth. The cytokinin-like activity of HS in stimulating the expansion growth of cotyledons has been demonstrated by earlier researchers [9] [10]. [14] showed several HS derived from leonardite and earthworm faeces or casting had cytokinin-like growth activity and contains physiological active concentrations of Isopentenyl adenine.

The variations in growth stimulatory effect on expansion growth of cotyledons can be explained in terms of differential amount and or potential of cytokinin-like growth stimulatory substance in HS formulations. The results clearly demonstrated that a few HS formulations have potential cytokinin-like influence on growth. HS formulations derived from compost sources were highly effective in stimulating cell expansion growth. The influence of HS formulation on cell elongation was examined using cucumber hypocotyl elongation bioassay. HS formulations derived from compost sources stimulated cucumber hypocotyl elongation growth, however, the extent of stimulation in growth differed between the formulations. HS derived from sedimentary sources were inhibitory; inhibitors co-extracted along with HS from sedimentary sources might be responsible for suppressing cell elongation resulting in growth inhibition.

Auxin and gibberellins are known to stimulate elongation growth of hypocotyl tissue [21] [22] [23]. HS were shown to display auxin like activity [24] [25]. Treatments enhancing endogenous auxins and gibberellin concentration were found to stimulate cucumber hypocotyl growth [26]. HS were shown to contain identifiable amounts of IAA [25], and gibberellins [13] [18] [19] [27]. Stimulated cucumber hypocotyl growth in response to HS formulations derived from compost source can be attributed to the presence of growth stimulatory substances similar to auxins and gibberellins. Recently, growth responses induced by HS have been studied at the molecular level. [28] remarked that HS elicit responses on most of the genes encoding synthesis and metabolism of plant hormones and expected to act on the main transcription factors and other regulatory genes in inducing growth response. Higher ratio of growth inhibitors to stimulators may be responsible for inhibitory growth observed in HS formulations derived from sedimentary sources. Leaf chlorophyll pigment is a functional component of chloroplast and helps in absorbing light energy and supplies for photochemical reactions of photosynthesis. It is an indirect indicator of photosynthetic activity and senescence. Dark and high temperature stress induces early senescence and chlorophyll degradation in detached leaves. HS are shown to induce chlorophyll synthesis and retards leaf senescence [29]. HS protect stability of chloroplast membrane under stress this may result in reduced breakdown of chlorophyll pigment content [30]. The results on chlorophyll pigment retention during stimulated senescence suggest that all HS formulations are effective in delaying chlorophyll degradation during senescence.

HS derived from both compost and sedimentary sources were found effective in reducing chlorophyll degradation. Formulations of HS derived from compost sources were more prominent in delaying chlorophyll pigment reduction during senescence. HS formulations J and I from compost are highly effective in delaying chlorophyll degradation. Humic substances mediated hormonal balance and high cytokinin-like influence may delay chlorophyll degradation during senescence. Hormone-like responses of HS on plants appear to be especially prominent for HS derived from compost [25]. [31] opined that humic acid extracted from composted manure is rich in carboxyl and phenolic compounds, making it more reactive and exhibiting higher complexion ability compared to humic acid from leonardite, and therefore it may serve as a promising alternative source for humic acid. Humic acid from manure or compost origin was found more effective in improving plant growth and plant nutrient uptake than humic and from coal [32] [33]. HS derived from compost and soils usually contains a low molecular weight distribution of molecules/aggregates, which has also been implicated in interactions with plant growth. The molecular size of HS also impacts their mode of action in plants as Low molecular size HS can readily enter root cells and directly elicit intracellular signaling, whereas high molecular size HS can bind to external cell receptors to induce molecular responses [17] [34]. The results from the present investigation prove that HS derived from compost sources have more effect than HS from sedimentary sources in stimulating growth at the cell level resulting in enhanced cell elongation and cell expansion processes.

5. Conclusion

Humic substances are recognized as one of the important biostimulant inputs to improve crop yield. HS are extracted mainly from sedimentary and compost sources and recommended for soil and foliar applications to improve crop growth and productivity. In this study, the direct biostimulatory effects of ten HS formulations, five each derived from sedimentary and compost sources were studied at the concentrations recommended for foliar applications. HS formulations showed wide variations in their direct biostimulatory influences. HS formulations derived from compost sources showed significantly more biostimulatory effect on cell expansion in cucumber and radish cotyledon growth expansion test compared to HS derived from sedimentary sources. Two of the five HS formulations stimulated cell elongation as shown by the cucumber hypocotyl elongation test. Formulations derived from sedimentary sources were not effective in stimulating hypocotyl elongation. All HS formulations were active in reducing chlorophyll degradation in cowpea leaf discs under accelerated senescence, HS formulations derived from compost sources were more effective in delaying chlorophyll degradation compared to HS from sedimentary sources. The results provided in this investigation clearly prove that HS derived from compost sources are more effective in directly influencing growth at the cell level than HS derived from sedimentary sources.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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