

Characterization of endophytic bacterial metabolites and their usage for biocontrol of *Colletotrichum falcatum* causing Red Rot in Sugarcane crop

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- The sugarcane crop provides a treasure trove of valuable products right from the intact canes to the crushing of crop. The sugarcane has established itself as an important cash crop and opened up new opportunity for agro-processing industry. However, sugarcane production does not correspond with the cultivated area in India. Several factors are responsible for low sugarcane productivity; among them, the major constraint in the successful crop production is the occurrence of several deadly diseases which not only affects its yield but also quality. Red rot is the key menace of sugarcane in India. The fungal pathogen *Colletotrichum falcatum* is responsible for causing devastating red rot disease in the standing crop.
- Numerous chemical methods have been employed for combating the disease; however, such methods failed as synthetic fungicides are resistant to degradation and their residue gets accumulated in the soil affecting the health of other beneficial microorganisms and their environment. The use of organically and biologically safe practices has drawn the attention of various eco-warriors, researchers, and scientists for manoeuvring such methods that are safe and eco-friendly especially using the microorganism through endophytic bacteria.
- Thus present study “**Characterization of endophytic bacterial metabolites and their usage for biocontrol of *Colletotrichum falcatum* causing Red Rot in Sugarcane crop**” was aimed to study endophytic bacteria and explore their multifarious metabolites that would contribute to red rot disease protection in sugarcane crop against fungal pathogen *C. falcatum*. Besides, protection to

disease resistance; various other growth-promoting and other traits were also explored for endophytic bacterial isolates.

- The three different cultivars namely Co 1148, CoJ 64 and CoS 767 of sugarcane were collected from the Indian Institute of Sugarcane Research (IISR), Lucknow and washed properly to remove the soil and processed for the isolation of endophytic bacteria. The culture plate of red rot pathogen i.e. *C. falcatum* (CF01) was also generously procured from the Division of Crop Protection, IISR-Lucknow.
- A total of 118 endophytic bacteria were isolated from the root and stalk tissue of sugarcane varieties (Co 1148, CoS 767, and CoJ 64). The variety Co 1148 yielded the highest number of endophytic bacterial colonies (59) followed by CoJ 64 (36) and CoS 767 (23) respectively from roots and stalk tissue. Isolation was done on different nutrient-rich media (LB, NA, PDA, KB, and LGIP media) and selective media (3% agar medium). The results for the isolation of endophytic bacteria also revealed that root tissue from all the cane varieties produces a higher number of distinct endophyte bacteria.
- After isolation of 118 endophytic bacterial isolates, they were checked for their *in-vitro* antagonism against *C. falcatum* on PDA medium. As antagonistic endophytic bacteria was the prime need of the study.
- Out of 118; only 29 isolated endophytic bacteria demonstrated *in vitro* inhibition against red rot fungus. It was further noticed that out of 29 isolates; a majority of strain (20) isolated from sugarcane variety Co 1148 showed inhibition against pathogen followed by variety CoJ 64 (7) and CoS 767 (2), respectively. Overall, only two strain B2 and B7 showed low *in vitro* inhibition with 47.57 ± 5.52 %

and $48.57 \pm 5.34\%$; while the remaining 27 endophytes showed more than 50% mycelial inhibition of fungal pathogen. Isolates S8, S11, S12 (from Co 1148), BS-4 (from CoS 767) and BS14, BS29 (from CoJ 64) showed maximum inhibition with $82.97 \pm 2.15\%$, $80.54 \pm 2.15\%$, $85.00 \pm 1.46\%$, $80.54 \pm 5.68\%$, $81.62 \pm 5.15\%$ and $80.54 \pm 2.15\%$, respectively.

- The selected 29 antagonistic endophytic isolates were also checked for another strong attribute i.e. sucrose tolerance (3-19%). The results revealed that all the twenty-nine endophytic bacterial isolates tolerated sucrose up to 11%. However, when further sucrose level was increased, only five isolates S8, S17, S12, R5, and BS-4 showed the highest tolerance level i.e. up to 19%.
- The morphological and biochemical characterization of all the twenty-nine endophytic isolates with antifungal activities were characterized. All the selected endophytic test isolates were distinct in their cultural characteristics. Morphological results revealed that nearly 79.31% of selected endophytes belonged to gram positive rod-shaped and rest belonged to gram-negative rod-shaped bacteria. All the 29 isolates were found to be motile. Various biochemical tests such as catalase, urease, IMViC, oxidase, and oxidation fermentation (O/F) were also conducted. Biochemically, sugars are very important for most of the microbe; therefore, carbohydrates utilization pattern were also recorded for all the twenty-nine isolates.
- After, morphological and biochemical characterization; the most efficient endophytic bacterial isolates were completely identified by using 16S rRNA sequencing analysis. Six endophytic bacteria (S8, S12, S17, S26, R5, and BS-4) based on *in vitro* antagonism, sucrose tolerant and other PGP characteristics

(discussed further) were selected for molecular identification. On the basis of 16 S rRNA sequencing; S8 was identified as *Bacillus cereus* with accession number (MG966498), S12 was identified as *B. aryabhatai* (MH298519), S17 as *B. subtilis* (MK411294), S26 as *B. licheniformis* (MK411295), R5 as *B. paramycooides* (MN318075) and BS-4 as *Pseudomonas aeruginosa* (MG966460).

- The 29 isolates along with antifungal property against *C. falcatum*; were further screened for the presence of various plant-growth-promoting and other antifungal metabolites or lytic enzymes in order to select the best isolates that possess multiple beneficial traits in the single isolate.
- The selected antagonistic endophytic bacteria when checked for their PGP traits, it was found that most of the bacteria had one or more PGP characteristics. IAA producing bacteria causes changes in the structure of root by stimulating root hair formation thus, ultimately increasing root surface area for mineral uptake and root exudation. In the current study; almost 51.72% (15 out of 29) of isolated endophytes showed IAA production. In present study, all the isolates produced different amount of IAA ranging from minimum 29.99 ± 2.59 $\mu\text{g/mL}$ in the isolate BS-4 to maximum 112.92 ± 4.16 $\mu\text{g/mL}$ in the isolate BS14.
- Endophytes are known to improve plant growth by phosphate solubilization after releasing organic acid, polysaccharides and the presence of phosphatase enzyme (Schachtman et al. 1998). In the present study, nearly 93.10% of isolated endophytes were found to have the potential to solubilize phosphorous. SI (Solubilization Index) of the isolates ranged from 1.20 ± 0.03 in isolate S22 to 2.67 ± 0.29 in isolate S26. Only two isolates i.e. B8 and B9 were not showing any halo zone. Rest all of the endophytes were showing positive confirmation for P-

solubilization. The quantitative estimation of available phosphate revealed that the highest amount of phosphate was present in BS-4 (272.85 μgPO_4^{3-}) and lowest in B12 isolate (11.28 μgPO_4^{3-}). Followed by BS-4; the higher amount of phosphate was also detected in the isolate R5, S-26, S17, and S8.

- Various species of *Bacillus* are known for IAA production and phosphate solubilization; but in the present study, entirely different species of *Bacillus*, which has never been reported in sugarcane and could be the novel strains i.e. *Bacillus aryabhatai* (S12) and *B. paramycooides* (R5) strain were found positive for these IAA and phosphate solubilization traits.
- All the 29 endophytic bacteria were showing positive results for ammonia production (another attribute that is linked with indirect plant growth promotion and used for biocontrol mechanisms). However, isolate S8, S17, R5, and BS-4 showed strong ammonia production after addition of Nessler's reagent that resulted in dark yellow to brownish colour.
- ACC deaminase; an another PGP trait that helps to alleviate the stress condition and screening result noticed that out of 29 endophytic bacteria only three (S8, S26, and R5) were found positive for the production of ACC deaminase as indicated by their growth on the DF medium.
- Besides, the above characteristics; HCN is an important PGP attribute and found that ten isolates viz. B2, B5, B8, B12, S11, S17, R5, BS-4, BS8, and BS15 endophytic bacteria showed positive test for HCN production as observed by change in the colour of filter paper from yellow to brown.

- The 29 endophytic bacterial isolates were also screened for siderophore production using the chrome azurol S (CAS) agar and only six bacteria namely S8, S12, S17, S26, R5 and BS-4 were found positive for siderophore production by change in colour of dye from blue to yellow- orange zones around the bacterial isolates in CAS-agar plates Further confirmation of these isolates for their siderophore presence in liquid assay was expressed in terms of percentage siderophore unit and it ranged from $10.58 \pm 4.24\%$ PSU in S26 to maximum $93.16 \pm 1.44\%$ PSU in BS-4. Therefore, the present study favoured the ability of siderophore production in appreciable amount by endophytic bacteria BS-4, S17 and R5, which are universally recognized biocontrol and plant growth promoting agents.
- Petri dish-based qualitative assays revealed that endophytic bacterial isolate produced variety of lytic enzymes such as chitinase, β -1,3 glucanase, protease, amylase, pectinase, and cellulase and that play an important role in lysis of fungal pathogen.
- Chitinase production was observed by a halo zone around the bacterial colony. The isolates S8, S12, S17, S26, R5 and BS-4 demonstrated the positive chitinase enzyme production. Similarly, S12, S17, R5 and BS-4 revealed the promising result for β -1, 3 glucanase enzyme production by formation of halo zone around the colony.
- Similarly, pectinase was observed in five isolates namely, R5, BS-4, S8, S12 and S17; caseinase enzyme was recorded in 23 isolates only; Cellulase, an important lytic enzyme responsible for degradation of cellulose was observed in the 16 isolates out of 29; while the amylase production was also found to be positive for

the 27 endophytic isolates. The isolates B1 and B7 did not show any amyolytic activity due to the absence of yellow zone around colonies after flooding the plate with iodine solution.

- As justified from the above findings that various attributes are present in endophytic bacteria that are responsible for revealing strong antagonism against red rot fungus. Besides, several other volatile and diffusible metabolites are also responsible for suppression of fungal pathogen. Therefore, *in vitro* analysis of volatile and diffusible compound were also conducted. It was revealed from the experiment that all the 29 antagonistic endophytic bacteria produced anti-fungal volatile compound(s) (VOCs) as evident from the growth inhibition of *C. falcatum*. In addition, aerial mycelial growth was also reduced due to the effect of volatile metabolites. Raza et al. (2016) demonstrated the role of VOCs produced by *Pseudomonas fluorescens* WR-1 in biocontrol activities. Kandel et al. (2017) and Lee et al. (2017) have also recently reported that VOCs mediate antifungal activities. Similarly, all the isolates were able to inhibit the mycelial growth due to the action of its diffusible metabolites.
- In order to evaluate the involvement of another putative antifungal compound in their suppressive effect, the antifungal activity of cell-free supernatant from the culture of all the 29 isolates were analysed. Cell-free supernatant of the isolates BS13, BS11, S21, S37 and B9 were able to inhibit the radial mycelial growth of *C. falcatum* with less than 50% inhibition. While rest all the isolates demonstrated strong inhibition against *C. falcatum* with highest in S12 isolates with 85.95%. These findings provide evidence that extracellular metabolites in the cell-free culture filtrate of isolates inhibited the growth of pathogenic fungi which prompted the further investigation of the effects caused by their

metabolites on the growth of the hyphal structure. There is a greater probability that the increased antifungal activity against the pathogen fungi tested by the culture filtrate of the isolates is a consequence of the production of extracellular secondary antifungal metabolites.

- Study of fungal-antagonist interaction from dual plate antagonism under simple microscopy revealed the distortion of hyphal structure in the growth of the test pathogen by all the endophytic strains. The isolates R5, BS-4, S8, S12, S26 and S17 were selected due to the possession of maximum PGP and antifungal lytic enzyme traits. Therefore, the antagonistic mechanisms of isolates S12, S8, S26, S17, R5 and BS-4 against hyphae of *C. falcatum* were also observed by scanning electron microscopy (SEM). These findings prompted the investigation that distortive effects (hyphal swelling, and distortion of mycelium) in pathogenic fungi, *C. falcatum* were due to the extracellular metabolites in the culture filtrates. Further, hydrolytic enzymes and secondary antifungal compounds may also be responsible for the distortion in the mycelium of the fungal pathogen.
- The present study filtered and selected the isolates R5, BS-4, S8, S12, S26 and S17 for future identification and application based on multiple beneficial traits.
- **Determination of Chitinolytic activity:** The enzyme activity of crude enzyme of six strain (R5, S12, S8, S17, S26 and BS-4) were investigated for the production of extracellular chitinase spectrophotometrically. The results revealed that all the six strain demonstrated chitinase production; however, the strain R5 exhibited maximum chitinase with 116 U mL⁻¹ followed by BS-4 and S17 isolate with 84 U mL⁻¹ and 79.4 U mL⁻¹, respectively at pH 7.0 and temperature 30° C. The isolate S26 detected low activity.

- **Determination of β -1,3 glucanase enzyme:** The qualitative test revealed the presence of β -1,3 glucanase enzyme in four isolates i.e. S12, S17, R5 and BS-4. Similarly, the activity of crude enzyme of the test isolates (S12, S17, R5, and BS-4) was evaluated for β -1,3 glucanase enzyme detection and the results revealed that R5 possessed the maximum enzyme activity with 415.8 U ml^{-1} followed by S17 and S12 with 385.3 U ml^{-1} and 225.1 U ml^{-1} respectively at pH 7.0 and temperature 30° C . While BS-4 detected low activity.
- **SDS and Zymogram of Chitinase activity:** Since the chitinase activity was found higher in five isolates viz. S8, S17, S12, BS-4 and R5 out of six. Therefore, the molecular weight of protein present in crude enzyme was assessed for five isolates S8, S17, S12, BS-4 and R5 only under SDS and it revealed various protein band profile. Chitinolytic zymography assay used in this study revealed a high resolution of single zymographic band for all the five isolates which had molecular weight of approximately $\sim 54 \text{ kDa}$ where lysis occurred. The zymogram study of crude chitinase revealed that activity was present in all the 5 isolates and band was approximately $\sim 54 \text{ kDa}$.
- **SDS and Zymogram of β -1,3 glucanase:** On the basis of qualitative and quantitative assay, strain R5, S12 and S17 were selected for characterizing the enzyme activity by electrophoresis and zymogram studies. The results for SDS-PAGE analysis revealed that several protein band were present in the crude supernatant detected after staining with Coomassie blue staining. However, single band of protein was observed as clear zone on the agarose gel by zymography. The band of S12 corresponds with size $\sim 54 \text{ kDa}$, molecular weight of protein

band for isolate S17 corresponds to ~60 kDa and that of R5 with ~ 59 kDa laminarin as substrate.

- **Detection of chitinase and β -1,3 glucanase gene in the isolates:**

Chitinase gene was screened for five isolates viz. S8, S17, S12, BS-4 and R5 and result revealed that only four isolates viz. R5, S8, S12 and BS-4 possessed the gene with approximately 300 bp amplicon. While β -1,3 glucanase gene with 300 bp was found in R5, S12 and S17 isolates.

- Further, the isolates R5, S12, S8 and BS-4 were selected on the basis of collective information on enzymatic activity, presence of gene and also due to the presence of various other metabolite. Therefore, the FTIR spectra of crude supernatant produced by endophytic isolates (R5, S12, S8 and BS-4) were analyzed and it revealed the presence of different bands corresponding to the following functional groups present in the molecular structure. Observed results demonstrated that FTIR spectrum of crude antifungal extracellular filtrate of isolates (BS-4, R5, S8 and S12) had characteristic peaks between 3746 - 698 cm^{-1} .
- **Identification of Antifungal compound by LC-MS/MS analysis:** Filter sterilized cell free extract of isolate BS-4, R5, S12 and S8 was also studied for bioactive antifungal compound by LC-MS/MS analysis. After 96 hr of bacterial growth, cell-free supernatants were extracted and analysis of crude extract of isolates BS-4 and R5 demonstrated the predominance of variety of metabolites as compared to S8 and S12 isolate.
- The antifungal metabolites from BS-4 extract in LC-MS/MS includes 1-hydroxy phenazine, Pyocyanin, phenazine 1- carboxylic acid (PCA), 2,4-

Diacetylphloroglucinol (DAPG), and Lahorenoic acid at positive mode. However, Siderophore (pyochelin), rhamnolipids, and presence of 4 hydroxy-2-alkylquinolines (HAQ) were present in negative mode.

- Similarly, crude extract of isolate R5 demonstrated the presence of multiple antifungal compound C-13, C-14, C-15 isomer of Surfactin, Bacillomycin, C-16 and C-14 isomer of Iturin, Fengycin A and cyclic lipopeptide Kurstakin.
- However, in comparison to R5 and BS-4 isolates; other two isolates S8 and S12 detected the presence of less lipopeptide antifungal compound. The crude extract of S12 revealed the presence of Kurstakin, Fengycin and Iturin only. The S8 crude extract showed the presence of C10, C11 Na adduct isomer of Kurstakin and Bacillomycin.
- **GC-MS analysis for detection of volatile compounds of selected isolates:**

The volatile organic compounds (VOCs) present in the crude extract of isolates BS-4, R5, S12 and S8 were identified by GC-MS analyses. The GC-MS chromatogram of ethyl acetate extracts of isolates BS-4, R5, S12 and S8 revealed the presence of various compounds with corresponding peaks at different retention times. Some of the common volatile antifungal compound in all the four crude extract of isolates found were Hexadecane, Pyrazine derivative, Dodecane, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), Dibutyl phthalate, Phenol, 2,4-bis(1,1-dimethylethyl)-, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl), 1,2-Benzenedicarboxylic Acid, Eicosane etc.

- **Efficacy to check bio-antagonistic endophytic bacteria under *in situ* condition:**

After analyzing all the necessary techniques to reveal the potential metabolites of the selected isolates, three endophytic bacterial strain *Bacillus paramycooides* (R5), *B. aryabhatai* (S12), *P.aeruginosa* (BS-4) and a mixture of *B. paramycooides* + *B. aryabhatai* (R5+S12) (as found compatible) were used for checking the bioefficacy against *C. falcatum* under *in vivo* conditions. The two isolates selected viz. *B. aryabhatai* (S12) and *B. paramycooides* (R5) were the novel endophytes in the sense that they have been reported from sugarcane and compatibility was found between these two isolates only, while, *P. aeruginosa* (BS-4) was selected due to the presence of multifarious metabolic compound.

- In the current study, four different methods/trials were adopted for checking the efficacy of bacterial endophytic strains for disease development in different trial setup: The four different trial were performed for the two consecutive years 2016-2017 and 2017-2018. The four trial (T-1, T-2, T-3 and T-4) were distinguished on the basis of differences in the inoculation of fungal pathogen *C. falcatum*; Trial-1 and Trial-4 had soil-based fungus inoculation in the sterile soil 15 days before sowing the setts while in the Trial-2 and Trial-3 pathogen was inoculated in the stalk and node region of cane far away from rhizosphere to induce the systemic resistance, respectively. In the trials (T-2, T-3 and T-4), the setts were sown after pre-treatment with the antagonistic endophytic bacteria followed by further application in the soil region and foliar spray in Trial-4. In the Trial-1, setts were planted without any prior treatments with endophytic bacteria and only two times rhizosphere application was followed after the plantation in a certain period.

- Although the effect of all the endophytic bacterial treatment either as sett or soil or foliar application resulted in the reduction of disease occurrence in the stalk of sugarcane. However, the efficiency of individual strain *B. paramycoides* strain R5 and *P.aeruginosa* BS-4 through sett or soil application effectively reduced the red rot incidence in all the trials and also promoted plant growth under field conditions better than the mixture /consortium of R5+S12. This finding is not in conformity with the work reported by other researchers (Molina-Romero et al. 2017; Bradáčová et al. 2019) while the finding is in conformity with the findings of Piakong and Zaida, (2018), who have reported efficacy of single strain over consortia.
- In the current study, the reason presumed for the low performance of mixture of R5+S12 in the *in vivo* condition over single strain R5 is either due to low efficiency of S12 strain in controlling the disease (less effective metabolites present) or due to lesser colonization in the root system for effective action which are essential requirement of bioagent to get into action against the disease resistance.
- The results suggested that the endophytic biocontrol agent *B. paramycoides* strain R5 significantly suppressed disease in the “stalk inoculation” experiments (where pathogen was inoculated above plant parts) i.e. in both Trial-2 and Trials-3, while the strain *P. aeruginosa* strain BS-4 exhibited best biocontrol activity in the “soil inoculation” experiments (where pathogen was inoculated in the soil) i.e. in the Trial-1 and Trial-4. The difference exhibited in the variation of the activity of two different strains i.e. *B. paramycoides* (R5) and *P. aeruginosa* (BS-4) in the four

different trial set-ups can be related to the variable metabolite production and their variable adoption mechanisms to suppress the fungal disease.

- Bacterized primed plants when challenged with a pathogen results in an induction and accumulation of a variety of defense-related proteins such as peroxidases (POX), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), total phenol, chitinases and β -1, 3-glucanases (Anand et al. 2007, Fishal et al. 2010). In the present study also, all the bacterial primed sett induced more resistance against red rot pathogen *C. falcatum* by induction of defense related enzyme. ISR can be elicited from the primed part to the distant part i.e leaves. Therefore, the experimental results of the present study demonstrated that endophytic mediated elicitation of systemic resistance in the sugarcane leaves against red rot disease leads to the development of a complex response involving the earlier and higher expression of defense enzyme like chitinase, β -1,3 glucanase, PAL, POX, PPO and phenol. Activity of the all the enzymes were found increased several times in the leaves of the bacterial inoculum treated sugarcane plants as compared to the control. Further, increase in the expression of these enzymes was much higher after inoculation of pathogen.
- Though there was an increase in the rapidity and the level of expression of all the enzymes; but Sugarcane leaves of Trial-2 and Trial-3 responded more prominently and rapidly by the accumulation of high levels of PAL, POX, PPO and phenolics due to pre-treatments of setts with endophytic bacterial followed by challenge inoculation with *C. falcatum*. However, in Trial-1 and Trial-4 where there was no challenged inoculation, active response were seen in the activity of all enzyme as compared to control. As setts in trial-4 were pre-treated with bacterial endophytes; rise and expression were more pronounced in Trial-4 as

compared to Trial-1. The present study was also consolidating with the study of Vivekananthan et al. 2004; Sundar et al. 2009.

- In the present investigation also, chitinase and glucanase activities significantly increased in all the endophytic treated cane leaves over control cane (Sugarcane). In the present study also, significant increased and highest activity was recorded in cane leaves treated with biocontrol agents R5 and BS-4. Thus, present study demonstrated the higher induction of the enzymatic system in susceptible variety treated with endophytic bacterial treatment.

- **Effects of Endophytic bacterial treatments on various parameters of cane growth:**

Percentage Germination: All the bacterial treated setts showed statistically significant increment in percentage germination when compared to control. In Trial-1, *P.aeruginosa* BS-4 resulted in highest germination of sett with 26.7% in the Year –I and in the Year-II *B. paramycoides* (R5) treatment resulted in highest germination with 28.8%. In Trial-2, highest germination was recorded in R5 with 37.8%. The other strains also exhibited significantly high germination percentage when compared with control. In Trial-3, R5 and BS-4 showed statistically equivalent germination with 42.2% followed by R5+S12. In the Trial-4 significantly higher germination was recorded for treatment with *P.aeruginosa* BS-4 followed by R5, R5+S12 and S12.

- In the present study, various other parameters such as cane height, shoot counts, cane weight, cane girth, number of internodes and chlorophyll content were recorded. From the present study; it was found that plant inoculated with bacterial endophytic bacteria demonstrated improved cane height, number of shoots, cane

weight, cane girth and number of internodes. The reasons for improving these physiological parameters might be due to the antagonistic endophytic strain with multifarious plant growth promotion which have been reported to enhance the growth in several ways. The PGPR strain have been demonstrated previously on their positive role in improving germination and cane growth (Viswanathan and Samiyappan 1999a, 2002).

- The efficiency of endophytic treated cane is also related to the performance of photosynthetic apparatus of the plant. Chlorophyll a, chlorophyll b and total chlorophyll contents were significantly higher in all the inoculated treatments compared with the control. Higher chlorophyll content in sugarcane treated with bacteria was also reported by Sevilla et al. (1998), Muthukumarasamy et al. (1999), and Chauhan et al. 2014.
- Therefore, the present study revealed the application of endophytic plant growth promotory bacterial strains as sett treatment followed by soil application could prove to be a beneficial component of integrated disease management as such combination resulted into higher accumulation of PR-protein and other defense enzyme response. These bacteria, apart from their action against pathogens are good growth promoters, which is an added advantage for any practical agricultural system.