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Biocontrol and plant growth promoting activity of bacterial strain *Pseudomonas aeruginosa* KUCd1in root rot disease of Chilli (*Capsicum* sp.) caused by *Phytophthora capsici* Leonian under *in vivo* conditions

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Phytophthora capsici is an important, destructive, broad-host range pathogens of Chilli (Capsicum annum L.) leading to high economic losses worldwide. Currently available control measures rely on excessive use of chemicals harmful to humans and the environment. Thus use of biocontrol agents can be considered as a safe and ecofriendly alternative to disease control. Pre-treatment with *Pseudomonas aeruginosa* KUCd1 strain reduced Phytophthora infection in chilli plants (62.5%). There was also considerable enhancement of various plant growth parameters such as root length (RL), root fresh weight (RFW), root dry weight (RDW), shoot length (SL), shoot fresh (SFW) and shoot dry weight (SDW) of the chilli plants in both winter and wet season trials. Increases were as follows: RL (27.3% in wet & 56.1 % in winter); RFW (42.6 % in wet & 29.8% in winter); RDW (39.6% in wet & 24% in winter); SL (18.3 % in wet & 16.3% in winter); SFW (3.8% in wet & 13.8% in winter) and SDW (5.5 % in wet &11.4% in winter,). KUCd1 was also a competent colonizer of the rhizosphere (6.32 log CFU g^{-1}) and rhizoplane (6.20 log CFU g^{-1}) in challenge inoculated chilli plants. Pretreatment of plants with plant growth promoting KUCd1 induced systemic resistance against *Phytophthora capsici* and elicitated rapid defence response (as evident from the increased activity of various defense-related enzymes; PAL, POD, PPO and catalases) protecting the plant from pathogen ingress and hence protection from disease. In conclusion KUCd1for its aggressive root colonization, plant growth promotion and biocontrol properties can be further explored as biofertizer/ biopesticide.

Key words: Chilli, PGPB, Biocontrol, *Phytophthora*

INTRODUCTION

Chilli (*Capsicum annuum*_L), also called red pepper, bell pepper belongs to the genus Capsicum, under the Solanaceae family. Chilli is one of the most important commercial crops of India and is grown almost throughout the country. However, Andhra Pradesh ranks first in area and production; Karnataka comes next to Andhra Pradesh closely followed by Orissa, West Bengal and Maharashtra. India is a major producer, exporter and consumer of chillies in the world. However,

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the average productivity is very low in comparison to that in other countries. Chilli is one of major solanaceous vegetable crops grown in West Bengal, (Chatterjee *et al*, 2011). Among diseases caused by fungi and fungi like organisms root rot of Chilli caused by *P. capsici* is a major limiting factor in increasing productivity in West Bengal as it is worldwide (Guha Roy *et al*, 2009; Naegele and Hausbeck, 2012).

Phytophthora capsici is an important plant pathogen of economic importance worldwide on many vegetable crops and infects more than 50 species in 15 plant families (Lamour, 2013). Among them *P. capsici* Leonian causes major disease of pepper and is found throughout production regions worldwide. The pathogen can infect pepper plants at all growth stages. *P. capsici* infects roots, crown, stems, leaves, and fruit, causing seedling death, stem lesion, leaf spot, foliar blight, and fruit rot. The first symptom on pepper in the field is commonly crown rot. Though root rot is the most common syndrome caused by *P. capsici* on pepper, foliar blight and fruit rot have also been identified. Fruit rot and foliar blight are less well studied and thought to be controlled by different genetic mechanisms than root rot. Phytophthora fruit rot can occur both pre- and post-harvest.

In fact, the management of most of the Phytophthora diseases relies on modifications in cultural practices, crop rotation, and judicious use of fungicides. The fact that fungicides against *Phytophthora* alone represent 25% of the total annual fungicide market is perhaps an indicator of the extent of damage caused and dependence on chemicals to control these diseases.

Changes in cultural practices, adjusting of planting times, disease forecasting and availability of resistant cultivars have improved disease management. However, fungicides like metalaxyl still remain an indispensable part of disease management programs and their routine use is neither economically practicable nor environmentally suitable. Moreover, fungicide-insensitive populations of *P. capsici* are becoming common and there are no fully resistant pepper cultivars with the desired horticultural type; field infestations can lead to total yield loss for multiple years. Fungicide resistance within pathogen populations and environmental recalcitrance is increasingly shifting the choice and need towards biological control agents suppressive to *P. capsici*.

Here we present data suggesting that *P. aeruginosa* KUCd1 could improve plant growth as well as reduce *Phytophthora* infection in brinjal under *in-vivo* conditions.

MATERIALS AND METHODS

Culture maintenance

The pathogen *P. nicotianae* isolate P10997, originally isolated from diseased tissue of chilli was maintained on V_8JA medium (Guha Roy *et al,* 2006). The information on host, accession number, morphological and genetic characterization and maintenance of the test isolate has been mentioned elsewhere (Guha Roy *et al,* 2006, 2007, 2009). The isolation and identification of bacterial agent KUCd1 as *Pseudomonas aeruginosa* following biochemical tests and 16S rDNA sequence analysis and its maintenance on nutrient agar (Himedia, India) has also been described in our earlier publication (Sinha and Mukherjee, 2008).

Plant material

The seeds of Chilli (*Capsicum annuum* L. var. Bullet) used for *in vivo* pot experiments were purchased from local nursery, Kalyani, Nadia, West Bengal. Before sowing, the seeds were surface sterilized with 1% sodium hypochlorite solution for 2 min, washed three times with sterilized distilled water (SDW), and blotted dry on sterile paper towels.

Bacterial inoculum

Sixteen hours grown bacterial culture of rifampicin resistant KUCd1 was transferred into nutrient broth (HiMedia, India) and incubated in a shaking incubator (160 rpm) at 28 ÚC for 24 h. The bacterial cells were harvested and centrifuged at 10000 rev min^{-1} for 20 min, washed twice and re-suspended in SDW. The final cell viable count was adjusted to ~1 × 10^8 CFU/mL before using as bacterial inoculant in the pot experiments. A rifampicin resistant (150 µg/mL) spontaneous mutants of KUCd1 (Sinha and Mukherjee, 2008) was used for root colonization studies.

Pathogen inoculum

Zoospore production was induced and isolated as

described by Ristaino (1990) from V_8JA maintained P10986 culture. The final concentration was adjusted to ~ 2×10^4 zoospores/ml and was applied immediately within 30 min of calibration.

Experimental set up

Plant growth and disease assessment studies for chilli plants were performed twice during winter (January) and wet season (July) in order to investigate the seasonal influence on disease incidence and plant growth in the polyhouse. The experiment comprised of four treatments; viz., T1 – water (control); T2 – KUCd1; T3– *P. nicotianae* (P10997); T4 – KUCd1 colonized plants challenged with P10997. Surface sterelized seeds of chilli were either soaked in SDW (T_1 and T_3) or in a KUCd1 cell suspension (~1×10^8 CFU/ mL, T_2 and T_4) for 24 h for bacterization followed by air drying. Seeds were sown in seedling trays and after two weeks the seedlings were transplanted into pots (38 cm diameter) containing potting mixture of solarised garden soil, farmyard manure and sand (3:2:1). Fifty millilitre of KUCd1 cell suspension (T_2 and T_4), prepared or SDW (T_1 and T_3) was applied by soil drench at the base of the stem of each chilli seedling immediately after transplanting. Two weeks after transplantation, the zoospore suspensions (T_3 and T_4) or SDW (T_1 and T_2) were injected at the rate of 5ml/per plant into 4 holes (1 cm diameter X 1cm deep) around each plant. Four seedlings were transplanted in each pot. There were four pots/ replication and 4 replications per treatment.The plants were covered with polythene bags for 24 h, continuously watered for 4-5 days to prevent drying of the soil and then watered as needed.

Plant growth and disease assessment

Chilli plants (4 weeks post inoculation) were uprooted, washed repeatedly in SDW to remove any adhering soil particles, and was blotted dry. The root and shoot fresh weight (g^{-1} plant) was recorded. These were then kept in the hot-air oven for 7 days at 60 ÚC for complete desiccation, and the dry weight (g^{-1}plant) was noted. The per cent mortality was calculated 30 days after pathogen inoculation.

Enzyme assay

The leaf samples were collected at 24 h, 48 h, 72 h, and 96 h post pathogen inoculation, washed with SDW and dried gently. The frozen leaf samples were homogenized in chilled 0.1 mol/L sodium phosphate buffer pH 7.0 with sterilized quartz sand and centrifuged at 15 000 rev min^{-1} for 20 min at 4 ÚC. The supernatant was stored at –80 ÚC. Protein concentrations of the samples were determined following Bradford's method (1976). Phenylalanine ammonia lyase (PAL*)* activity was assessed following the method as described by Zucker (1965) by measuring the amount of cinnamic acid produced at 290 nm and is expressed as U mg^{-1} protein. Peroxidase (POD) activity was determined following the method of Urbanek *et al*. (1991) and is expressed as µmol/l H_2O_2 min^{-1} (1 unit) g^{-1} protein taking into consideration that 4 mol/l of H_2O_2 are reduced to produce 1 mol/l of tetraguaiacol. Polyphenol oxidase (PPO) activity was determined following standard method (Mukherjee and Ghosh, 1975) and is expressed as increase in absorbance at 420 nm min^{-1} mg^{-1} protein. Catalase (CAT) activity was estimated and was expressed as $unit^{-1}mg^{-1}$ protein. The reaction mixture consisted of 3 ml of hydrogen peroxide-phosphate buffer and 0.03 ml enzyme extract. The reaction mixture was shaken well and absorbance value at 240 nm was noted immediately at interval of 10 or 20 s. The time required for decrease in absorbance from 0.45 to 0.40 was noted.

Root colonization bioassay

Plants were uprooted at 60 days after sowing for rhizosphere and rhizoplane bacterial population study.The excised roots from these uprooted plants were vigorously shaken to collect the adhering rhizosphere soil particles. These particles were then mixed thoroughly on a sterile filter paper and 10 g of this mix was put into a 250 mL Erlenmeyer flask containing 100 ml of SDW. In order to estimate the rhizoplane bacterial population, the root segments were cut into small pieces, mixed randomly and 10 g of this mix was washed three times in SDW and homogenised with sterile mortar and pestle. These homogenised roots were placed in a 250 mL Erlenmeyer flask filled with 100 ml of sterile water. The flasks were shaken at 160 rpm at 28 ºC for 30 min. The suspension was spread and cultured at 28 ºC for 48 h on NA medium supplemented with 150 µg/ml

Statistical analysis

The data were statistically analyzed using the SPSS

software, version 17. Analysis of variance (ANOVA) was determined using general linear model and mean values were compared by Duncan's multiple range test (DMRT) at 5% probability. The standard error of mean (SEM) for each treatment was calculated.

RESULTS AND DISCUSSION

This experiment investigates the growth promoting and disease suppressing ability of *P. aeruginosa* strain KUCd1 against the Phytophthora root rot disease of chilli.

In the seasonal trials, bacterized sets (T_2 and T_4) demonstrated significantly higher values for all the measured growth parameters compared to non-bacterized sets (T_1 and T_3) at *p* d" 0.05. The results further indicated that irrespective of the seasonal variation, T_2 plants, accounted for the maximum increase in growth parameters compared to all other treatments. In T_4 although the percent increase in growth parameters was lower compared to that recorded in T_2, however, it still registered significantly (*p* d" 0.05) higher values compared to T_1 and T_3 in the test plants.

The data for both winter and wet trials has been summarized in Table 1. During winter trial, the increase in different growth parameters of T_2 plants over control (T_1) was noted to be maximum for root length (56.1 %) followed by root dry weight (42 %), root fresh weight (33.3 %), shoot length (28.8 %), shoot fresh weight (19.1 %) and shoot dry weight (14.6 %). In T_4 plants this increase in growth parameter over control was reduced compared to T_2 plants. The maximum increase was observed in case of root fresh weight (29.8 %) followed by root dry weight (24 %), shoot length (16.3 %), shoot fresh weight (13.8 %) and shoot dry weight (11.4 %). The results also indicated reduction in root length compared to control set by 9.09 % in winter trial (Fig. 1).

The result clearly demonstrated that changes in growth parameters irrespective of seasonal variations in all KUCd1 treated sets applied alone (T_2) or in combination with P10997 (T_4) were significantly higher than the untreated ones, i.e., the control sets (T_1) and only P10997 treated sets (T_3). The only exception being decrease in root length in T_4 (KUCd1 + P10997) sets. But it was observed that there was increment in the other two growth parameters of root, viz., root fresh weight and root dry weight. This increment can be attributed to enhanced lateral growth of root observed in pre-treated plants.

The disease control efficiency of the proposed biocontrol agent KUCd1 for root rot diseases of chilli was calculated at 30 days post challenge inoculations with *P, capsici* (P10997). Chilli plants challenge inoculated with P10997 (T_3) showed 100% mortality of plants. No plant mortality was observed in either T_1 or T_2 sets. The T_4 sets showed reduced mortality rate compared to T_3 set. KUCd1 reduced mortality in Chilli by 62.5 % (Fig. 2).

The results of the above study clearly indicate that KUCd1 is an aggressive root colonizer which corroborates our earlier findings (Sinha and Mukherjee, 2008). It improved plant growth parameters in chilli and also had a protective effect against *P. capsici* under *in vivo* controlled conditions which is in line with *P. aeruginosa* strains such as 7NSK2 (Buysens *et al.,* 1996), PNA1 (Anjaiah *et al.,* 2003), NJ-15, PUPa3 (Sunish Kumar *et al,* 2005), GRP3 (Sharma *et al.,* 2007), Sh8 (Siddique and Meon, 2009), RM-3 (Minaxi and Saxena, 2010), FP6 (Bakthavatchalu *et al.,* 2012), PGPR2 (Illakkiam *et al,* 2014) that have been reported to have plant growth promoting and biocontrol activity against phytopathogens.

Pre-treatment with KUCd1 also elicited resistance in chilli plants by induction of host defense-related enzymes PAL, POD, PPO and catalase before inoculation with pathogen (P10997). However, there was further increase in enzymatic activity during post pathogen inoculation. The result obtained are in agreement with those of Siddiqui and Meon (2009) who observed similar increase in activities of defense-related enzymes in chilli plants whose seeds were bacterized with *P.aeruginosa* UPMP3 and UPMB3.

The high POD activities detected in treatments are linked to lignifications and generation of hydrogen peroxides that inhibit pathogens directly or generate other free radicals with antimicrobial effects (Chen *et al*, 2000). The role of PPO in plant defense responses is also well known and like POD mediates the oxidation of phenols. Induction of POD, PPO and PAL and accumulation of lignin and phenolic compounds to reinforce plant cell walls, have been correlated with disease resistance in a

Table 1. Effect of promising antagonistic isolate KUCd1 on growth performance of Chilli under artificial infestation of pot soils with P10997 in net house, 60 days after sowing, during winter and wet trials

Treatment[+]	Growth Parameters[*](Winter Trial)						Growth Parameters[*] (Wet Trial)					
	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)
Control (T_1)	11 b (± 0.33)	1.68 b (± 0.01)	0.5 c (± 0.01)	38.83 c (± 0.42)	13.52 c (± 0.15)	3.15 b (± 0.05)	13.75 c (± 0.31)	1.69 c (± 0.02)	0.53 c (± 0.01)	40.58 c (± 0.70)	21.36 c (± 0.10)	3.98 c (± 0.03)
KUCd1(T_2)	17.17 a (± 0.53)	2.24 a (± 0.03)	0.71 a (± 0.01)	50 a (± 0.48)	22.05 a (± 0.19)	3.61 a (± 0.06)	17.5 a (± 0.62)	2.64 a (± 0.06)	0.78 a (± 0.01)	53 a (± 0.48)	23.71 a (± 0.13)	4.45 a (± 0.06)
P10997 (T_3)	6.33 c (± 0.31)	0.75 c (± 0.01)	0.25 d (± 0.01)	27 d (± 0.39)	8.42 d (± 0.01)	1.67 c (± 0.01)	10.25 d (± 0.35)	0.91 d (± 0.01)	0.3 d (± 0.01)	29.67 d (± 0.62)	10.22 d (± 0.20)	1.73 d (± 0.02)
KUCd1+P10997 (T_4)	10 b (± 0.39)	2.18 a (± 0.04)	0.62 b (± 0.01)	45.17 b (± 0.42)	21.08 b (± 0.11)	3.51 a (± 0.06)	12 b (± 0.25)	2.41 b (± 0.05)	0.74 b (± 0.01)	48 b (± 0.58)	22.16 b (± 0.16)	4.2 b (± 0.04)

+ Plant treated with KUCd1 were challenge inoculated with *P.capsici* (P10997). Untreated plants served as negative control when exposed to water treatment while plants exposed to pathogen served as positive control. Data are the means (± SEM, standard error of means) of two separate experiments with 12 plants per treatment per experiment. Means followed by different letter in a column are significantly different according to Duncan's multiple range test (DMRT) at *p* d" 0.05.

number of plant–pathogen interactions (Chen *et al*, 2000; Mohammadi and Kazemi, 2002). Reports of increases in PAL and its relation to resistance to pathogen infection in plant tissues or triggering of ISR pathways in bean roots have been cited by earlier workers (De Meyer and Höfte, 1997; Van Wees, 1999). In the present study too, bacterial treatments resulted in significant increase in both PAL and POD levels in chilli plants which is in conformity with the above findings. It was further noted that the catalase activity was high in KUCd1-pretreated chilli plants challenged with P10997 while in plants inoculated with pathogen (P10997) alone

Table 9. Population dynamics of *Pseudomonas aeruginosa* strain KUCd1 on the rhizoplane and rhizosphere of Chilli, in pot trials 30 days after inoculation

Treatment	Rhizoplane population (log cfu g^{-1})	Rhizoplane population (log cfu g^{-1})
KUCd1	7.65	7.45
KUCd1+ Patho*	6.45	6.20

* Plants treated with *Pseudomonas aeruginosa* strain KUCd1 were challenge inoculated with respective pathogens- P10997 (*Phytophthora capsici*) in Chilli; P10986 (*Phytophthora nicotianae*) in Brinjal and 35B9 (*Phytophthora colocasiae*) in Taro cultivars (BCC1 & Telia).

Fig. 1 : Effect of KUCd1 on induction of phenylalanine ammonia lyase (PAL) in root (A), stem (B). leaves (C); and peroxidase in root (D), stem (E), leaves (F) of chilli plants during the experimental period otf 24 h to 96 h post *Phytophthora capsici* P10997 inoculation. The four treatments are represented by T1- control (non-inoculated and non-bacterized), T2- KUCd1 treated, T3- P10997 treated and T4- KUCd1+P10997 treated. Means followed by the same letter are not significantly different between treatments according to Duncan's multiple range test(DMRT) at *p≤0.05.* Bars represent(±)SE of mean.

increased initially but then decreased. Similar findings were reported in *P. fluorescens*-pretreated cucumbers challenged with *Pseudoperenospora cubens* and *Erisiphe cichoracearum,* and *Brassica* species in the initial stages of infection by *Alternaria* which makedly dropped at later stages; in *P. aureofaciens* 63-28 pretreated soybean challenged with *R.solani*; in pepper roots challenged

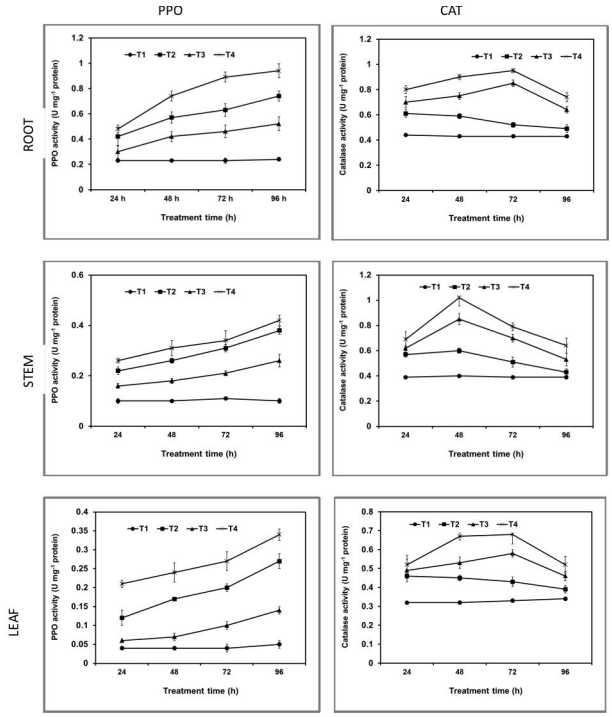

Fig. 2 : Effect of bacterial agent *P. aeruginosa* KUCd1 on induction of polyphenol oxidase (PPO) in root (A), stem (B), leaves (C); and catalase in root (D), stem (E), leaves (F) of chilli plants during the experimental period of 24 h to 96 h post *Phytophthora capsici* P10997 inoculation. The four treatments are represented by T1- control (non-inoculated and non-bacterized), T2- KUCd1 treated, T3- P10997 treated and T4- KUCd1+ P10997 treated. Means followed by the same letter are not significantly different between treatments according to to Duncan's multiple range test(DMRT) at $p \leq 0.05$. Bars represent (±) SE of mean.

inoculated with *P. capsici*. Therefore, this increase in peroxidase, polyphenol oxidase, catalase and phenylalanine ammonia lyase activities in tissues of chilli plant can be interpreted as an inducible defense mechanism used for protection against pathogen invasion through lignin deposit at the plant cell wall.

Microbial induced defense in plants may present differential level of efficiency in suppressing pathogen attack depending on the ability of the pathogen to avoid activated host defense. However, rhizobacteria isolate with ISR have increased chances of success in suppressing disease caused by a broad spectrum of pathogen than biological control agent with single biocontrol mechanism Thus success of KUCd1as a biocontrol agent in the field may be attributed to the repertoire of arsenals that have been detected to have HCN and siderophore producing ability. Both HCN and high levels of siderophores (Sinha and Mukherjee, 2008) production by KUCd1 can play an important role in inhibition of phytopathogenic oomycetes by depriving them of this essential element since the siderophores from pathogen have lower affinity (O'Sullivan and O'Gara, 1992; Loper and Henkel, 1999) and the bacterial siderophore-iron complexes can be exploited by plants (Sharma and Johri, 2003). Strains of *P. aeruginosa* has also been shown to induce systemic resistance, cause disease reduction and/or promote plant growth in bean, rice, tomato and chilli (De Meyer *et al*, 1999; Audenaert *et al,* 2002; Buysens *et al*, 2006; Saikia *et al*, 2006; Sharma *et al*, 2007).

Microbial production of HCN has been reported as an important antifungal trait to control root infecting fungi (Ramette *et al*, 2003) and disease suppression due to production of hydrogen cyanide was reported earlier (Voisard *et al*, 1989; Ahmad et al, 2008). It was considered that HCN was the main mechanism of action and its expression and production by *Pseudomonas* was believed to be strongly dependent on iron availability (Keel *et al*, 1989; Voisard *et al*., 1989; Blumer and Haas, 2000). HCN inhibits the terminal cytochrome c oxidase in the respiratory chain and binds to metalloenzyme (Bakker and Schippers, 1987). The antifungal effect of *Pseudomonas* was thought to be due to the production of HCN and siderophore or synergistic interaction of these two or with other metabolites (Ahmad *et al*, 2008). KUCd1 being a producer of HCN might as well have used this trait to suppress plant disease in the various plant–pathogen interactions under study. It was also found that the beneficial effect on plant shoot dry mass was more pronounced with HCN producing *Pseudomonas* strain in studies conducted by Gol *et al*, (2002). Therefore, KUCd1 that happens to have many of these biocontrol traits was able to make use of one or more of these mechanisms to reduce the growth of the pathogenic *Phytophthora* sp. (Guha Roy, 2007) while inducing plant growth.

In conclusion, the present study demonstrated primarily the *P. capsici* antagonistic and plant growth

promoting property under controlled *in vivo* conditions amongst the other multifunctional properties of *P. aeruginosa* strain KUCd1. The said bacterial strain had a broad antagonistic activity which helps in establishing and resisting against deleterious microorganisms occupying the microbial niche in the rhizosphere particularly the oomycetous plant-destroyer, *P. capsici*. Further siderophore production and antimicrobials released by the strain reflect their rhizospheric competitiveness that can be beneficially combined with plant protection and its PGPR traits to help enhance the plant growth. These together with resistance to cadmium toxicity and other heavy metal tolerance could prove to be an added advantage in able to survive in soil containing high concentration of these metal ions and still carry out its biocontrol and PGPR function while playing a significant role in bioremediation as well. However, field evaluation is necessary to determine its efficacy under natural ecosystem.

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