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Original Research Paper

Piriformospora indica and *Bacillus velezensis* reduced titres of PRSV and CMV and suppressed symptoms of snake gourd mosaic disease

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ABSTRACT

Pre-endophytic colonization of snake gourd (*Trichosanthes cucumerina* L.) with *Piriformospora indica* (1% w/v), followed by individual pre-colonizations with *Bacillus velezensis* (10^8 CFU mL^{-1}) and arbuscular mycorrhizal fungi (AMF; 1% w/v), significantly mitigated the incidence and severity of snake gourd mosaic disease under laboratory and field conditions. The endophytic treatments effectively suppressed the titres of *Papaya ringspot virus* (PRSV) and *Cucumber mosaic virus* (CMV), as confirmed by DAC-ELISA and DAS-ELISA analyses. Among the treatments, *P. indica* pre-colonization resulted in the lowest viral load and delayed symptom expression, followed by *B. velezensis* and AMF. Enhanced activities of defense-related enzymes viz., peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, catalase and ascorbic acid oxidase were recorded in *P. indica*-colonized plants, indicating the induction of systemic resistance. SDS-PAGE analysis revealed the induction of a novel 31.57 kDa protein in AMF-treated plants, while, *P. indica* and *B. velezensis* colonization altered overall protein profiles relative to the control. Field evaluations demonstrated a marked reduction in disease incidence (88.36% in *P. indica*, 92.95% in *B. velezensis* and 99.74% in AMF treatments at 90 DAS), improved vegetative growth and yield, with *P. indica* recording the highest yield (7.04 kg $plant^{-1}$).

Keywords: CMV, ELISA, endophytes, *Piriformospora indica*, PRSV, snake gourd mosaic

INTRODUCTION

Snake gourd (*Trichosanthes cucumerina* L.) is a highly nutritious vegetable belonging to the family Cucurbitaceae, extensively cultivated across Asia. The plant is known to exhibit medicinal properties like anti-inflammatory and anti-diabetic effects (Atugwu et al., 2022). However, the crop is vulnerable to various viral diseases, which manifest as mosaic symptoms and result in yield losses. In India, the mosaic disease affecting snake gourds has been associated with viruses from the genera begomovirus, potyvirus and cucumovirus. The endophytic microorganisms which are found within plant tissues have the potential to be utilized as biocontrol agents against such mosaic disease which is a reliable and eco-friendly approach (Farhana et al., 2024). Plants treated with *Bacillus* spp., a bacterial endophyte have showed enhanced resistance against Cucumber mosaic virus (Elsharkawy et al., 2022) and Tobacco mosaic virus (El Gendi et al., 2022). Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs and there are reports that plants colonized with mycorrhiza showed resistance against Tomato mosaic virus (Aseel et al., 2019) and Cardamom mosaic virus (Vijesh, 2023). *Piriformospora indica* is a beneficial and axenically cultivable root colonizing endophytic fungus and plants primed with *P. indica* showed enhanced resistance against *Cowpea aphid borne mosaic* (Alex, 2017) and *Blackeye cowpea mosaic virus* (Chandran, 2019). In this context, the study aims to evaluate the efficiency of beneficial bacterial endophyte *Bacillus velezensis* and fungal endophytes *viz.,* AMF and *P. indica* for the management of snake gourd mosaic disease under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Maintenance of virus inoculum

Snake gourd samples showing mosaic, mosaic mottling, vein banding, blistering, distortion, puckering and deformed fruits were collected from snake gourd fields at College of Agriculture, Vellayani,

Thiruvananthapuram *via* purposive sampling method. The viruses were maintained in snake gourd variety Kaumudi under insect proof conditions through mechanical transmission. Reverse transcription polymerase chain reaction was carried out with primers specific to the coat protein of PRSV and 2a protein of CMV (Kumar et al. 2014; Kumari et al. 2021) for the molecular diagnosis of viruses infecting snake gourd plants.

Co-cultivation of endophytes

Pure cultures of the bacterial endophyte *Bacillus velezensis* PCSE 10 and the fungal endophytes *Piriformospora indica* and a consortium of arbuscular mycorrhizal fungi (AMF) comprising *Glomus fasciculatum*, *G. mosseae* and *G. etunicatum* were obtained from the Department of Microbiology, College of Agriculture, Vellayani. All cultures were maintained aseptically in their respective growth media (nutrient agar and potato dextrose agar) to preserve purity and viability.

The fungal endophyte *P. indica* was cultured in potato dextrose broth (PDB) adjusted to pH 6.5 and dispensed into 500 mL Erlenmeyer flasks. The flasks were incubated at 25±1°C in an orbital shaker at 120 rpm for 14 days to promote uniform mycelial growth. Following incubation, the fungal biomass was harvested by filtration through sterile muslin cloth and washed thoroughly with sterile distilled water to remove traces of medium. The inoculum was prepared at a final concentration of 1% (w/v) using a sterile carrier mixture of vermiculite and coir pith (2:1 ratio), following the procedure described by Anith et al. (2011).

The bacterial endophyte *B. velezensis* PCSE 10 was cultured in nutrient broth at 30±1°C for 48 hours under continuous shaking at 150 rpm. The resulting culture was standardized to a cell density of 1×10^8 colony-forming units (CFU) mL^{-1} using spectrophotometric calibration and serial dilution techniques.

For the purpose of inoculation, seeds of snake gourd variety 'Kaumudi' were surface-cleaned and soaked in sterile distilled water for 8 hours to enhance germination. The pre-soaked seeds were sown in earthen pots containing a sterilized potting mixture of sand, soil and well-decomposed cow dung in a 1:1:1 (v/v/v) ratio. Pots were maintained under insect-proof glasshouse conditions (28±2°C; 70–80% relative humidity) to prevent external contamination and vector-mediated virus transmission.

For AMF and *P. indica* treatments, five grams of vermiculite-based AMF inoculum and 1% (w/v) *P. indica* inoculum were incorporated into the potting mixture prior to sowing. Seeds were sown after a 4-hour priming period. For bacterial inoculation, soil drenching was performed using *B. velezensis* cell suspension (1×10^8 CFU mL^{-1}), followed by foliar spraying of the same suspension at 10-day intervals after seedling emergence. In the combined treatment, all three endophytes namely *P. indica*, *B. velezensis*, and AMF were applied simultaneously following the respective inoculation protocols. Uniform agronomic and moisture management practices were maintained for all treatments to ensure optimal seedling establishment and endophytic colonization. Root colonization by AMF and *P. indica* in snake gourd plants was confirmed following the staining procedure of Phillips & Hayman (1970) with modifications by Johnson et al. (2013). Roots from each treatment were thoroughly washed to remove adhering soil particles, cut into 1 cm segments and cleared in 10% (w/v) potassium hydroxide (KOH) at 65°C for 5 minutes. The cleared roots were rinsed, acidified in 1% (v/v) hydrochloric acid (HCl) for 5 minutes and stained with lactophenol trypan blue to visualize fungal structures. Excess stain was removed and roots were destained in lactophenol to obtain clear visibility. The stained root segments were mounted on glass slides and examined under a compound microscope (Olympus CX43) at 400X magnification. The presence of arbuscules, vesicles and hyphae in AMF-treated roots, and chlamydospores of *P. indica* within the root cortex, confirmed successful endophytic colonization.

Pot culture experiment

The layout for the pot culture experiment was a completely randomized design with six treatments and three replications with five plants per replication. The six treatments were *B. velezensis*+virus; AMF+virus; *P. indica*+virus; *B. velezensis*+AMF+*P. indica*+virus; virus alone and absolute control.

Freshly made virus inoculum was mechanically inoculated (sap transmission) to plants at three leaf stage and observations *viz.*, days taken for symptom development, nature of symptoms and number of plants infected were made. All the colonized and control plants were assessed for the presence of CMV and PRSV with ELISA using polyclonal antibodies at 40, 50 and 60 DAS (days after sowing), *i.e.* Double

antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) with PRSV polyclonal antiserum (DSMZ GmbH, Braunschweig, Germany) for PRSV and direct antigen coating ELISA (DAC-ELISA) with CMV polyclonal antiserum for CMV (ICAR-NRC for Banana, Tamil Nadu).

Estimation of induced proteins

Electrophoretic separation of soluble protein from snake gourd leaves of best three treatments identified from pot culture studies were carried out using Sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) at 60 DAS as per the procedure described by Laemmli (1970).

Field studies

The three promising treatments selected from pot culture studies *viz*., individual pre-endophytic colonizations with *P. indica*, *B. velezensis* PCSE 10 and AMF were evaluated in field conditions under natural incidence of snake gourd mosaic disease along with control plants. Seeds were pre-soaked in water and sown in the field. *B. velezensis* (10^8 CFU per mL) was used for seed priming, soil drenching and foliar spraying on snake gourd at three leaf stage. AMF and *P. indica* 1 per cent (w/v) inoculum were applied in small pits over which the seeds were sown directly. The three promising endophytes were applied separately as pre colonization treatments and control plants were also kept. Plants were monitored for disease incidence and observations were made at regular intervals. The layout for the experiment was randomized design using 4 treatments (15 plants per treatment) and 5 replications (3 plants per replication) with a total of 60 plants. Observation for plant bio-metric parameters, symptom development and incidence of snake gourd mosaic disease were made at 30, 45 and 60 DAS.

RESULTS AND DISCUSSION

Maintenance of virus inoculum

On mechanical transmission of virus to healthy snake gourd plants, typical mosaic symptoms appeared after 14 days of inoculation with 100 per cent transmission; and disease score chart was prepared (Fig. 1). Snake gourd varieties *viz.,* TN TNAU SG1 and TN TNV SG1 were successfully inoculated with CMV by Nagendran et al. (2018) through mechanical transmission using 0.1 M sodium phosphate buffer (pH 7.0).

RT-PCR using specific virus specific primers of PRSV and CMV, gave amplicons of sizes 850 and 450 bp, respectively, thus, detecting viruses from infected samples. Asok et al. (2024) reported that infected snake gourd samples reacted positively to primers specific to PRSV and CMV, yielding amplicons of sizes 1200 bp and 400 bp, respectively.

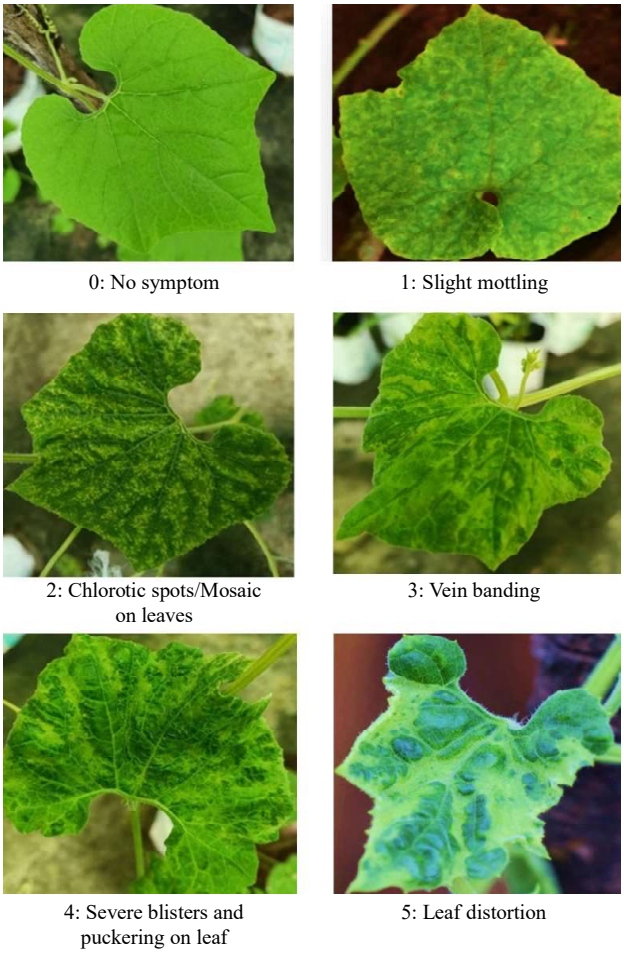


Fig. 1 : Score chart used for snake gourd mosaic disease

Co-cultivation of endophytes

Successful root colonization of snake gourd plants with endophytes was observed at 30 days after inoculation. Chlamydospores of *P. indica* were observed in cortical and epidermal layers of roots upon staining with trypan blue. *G. fasciculatum* colonization was confirmed by the presence of arbuscules and vesicles inside the plant cell. Subhash et al. (2025) detected the presence of chlamydospores of *P. indica*, along with vesicles and arbuscles of arbuscular mycorrhizal fungi, through trypan blue staining in excavated cassava roots.

Table 1 : Effect of endophytic colonisation on development of snake gourd mosaic disease incited by PRSV and CMV

Treatment	No. of days taken for symptom development	40 DAS		50 DAS		60 DAS	
		Disease incidence (%)	vulnerability index	Disease incidence (%)	vulnerability index	Disease incidence (%)	vulnerability index
B. velezensis + virus	20.000 ± 1.000[b]	33.33	10.60	46.66	18.67	46.66	64.00
AMF+ virus	21.000 ± 1.000[b]	40.00	13.32	60.00	29.33	60.00	76.33
P. indica + virus	22.667 ± 0.577[a]	20.00	9.30	33.33	14.60	33.33	48.00
B. velezensis + AMF + *P. indica* + virus	17.667 ± 0.577[c]	53.33	20.00	66.66	37.33	66.66	86.66
Virus alone	14.333 ± 0.577[d]	73.33	30.00	80.00	65.30	100.00	92.00
Absolute control	Nil	0.00	0.00	0.00	0.00	0.00	0.00
SE	0.447						
CD	1.409						

Values are mean of 3 replications ± standard deviation; SE: standard error; C.D: critical difference; **values in parenthesis are *arc sine* transformed; DAS: days after sowing

Pot culture experiment

Among the colonized plants, those colonized with *P. indica* exhibited a higher number of days for symptom expression (22 days), followed by those colonized with AMF and *B. velezensis*. *P. indica*-colonized snake gourd recorded a reduced vulnerability index (9.30), followed by *B. velezensis* (10.60) and AMF-colonized plants (13.32) against control plants (30.00) at 40 days after treatment (DAT).

P. indica colonized snake gourd plants showed higher efficiency in managing the virus disease as the colonization resulted in conspicuous delay in virus symptom expression (22 days) compared to control plants (14 days). *P. indica* colonization resulted in a lower disease incidence (33.33 per cent) as well as lower disease severity (48.00) at 60 DAS in comparison with virus infected non colonized control (disease incidence 100 per cent), vulnerability index 92.00) (Table 1). Ramseena (2023) reported that seed priming, foliar spray and soil drenching of *B. pumilus* VLY17 cell suspension at the cotyledonary leaf stage of cowpea plants inoculated with Tospovirus 5 DAC recorded a lesser disease severity (26.6), with lowest disease score of 1 compared to control plants.

In the present study, compared to un-colonized control plants, endophyte colonized snake gourd plants upon immunological diagnosis showed lower titre of CMV and PRSV at 40, 50 and 60 days after sowing (Table 2 & 3). *P. indica* colonized snake gourd plants gave the least fold increase in OD value over absolute control for CMV (2.07±0.01) and PRSV (2.15±0.01) followed by *B. velezensis* (2.16±0.02, 2.46±0.02) and AMF (2.56±0.02, 2.84±0.04) at 60 DAS. Likewise, Lekshmi (2021) recorded that *P. indica* colonized black pepper plants post inoculated with PYMoV showed least virus titre compared to control plants.

Estimation of induced proteins

SDS PAGE revealed that, five proteins each were profiled from plant samples pre colonized with *P. indica* (80.86, 52.83, 45.06, 35.53 and 17.39 kDa) and those pre colonized with *B. velezensis* PCSE 10 (75.61, 51.67, 43.87, 34.82 and 17.244 kDa) (Fig. 2). Pre colonization with *P. indica* and *B. velezensis* PCSE 10 reduced the number of profiled proteins compared to control plants. This may be due to activation of host proteasome activity and extracellular proteases, enhancing the degradation of viral proteins during infection, which resulted in a

Table 2 : Effect of colonization of endophytes, *B. velezensis, P. indica* and AMF individually and combination on CMV in snake gourd plants of var. Kaumudi at 40, 50 and 60 DAS

Treatment	Fold increase in OD value over absolute control		
	40 DAS	50 DAS	60 DAS
B. velezensis + virus	0.56 ± 0.02[d]	2.18 ± 0.03[d]	2.16 ± 0.02[d]
AMF + virus	0.70 ± 0.02[c]	2.58 ± 0.03[c]	2.56 ± 0.02[c]
P. indica + virus	0.40 ± 0.02[e]	2.05 ± 0.02[e]	2.07 ± 0.01[e]
B. velezensis + AMF + *P. indica* + virus	0.90 ± 0.02[b]	2.95 ± 0.02[b]	2.97 ± 0.02[b]
Virus alone	2.63 ± 0.15[a]	4.58 ± 0.03[a]	4.98 ± 0.03[a]
SE (m)	0.04	0.01	0.01
C.D	0.13	0.04	0.03

Values are mean of 3 replications ± standard deviation; SE: standard error; C.D: critical difference; DAS: days after sowing

Table 3: Effect of colo nization of endophytes, *B. velezensis, P. indica* and AMF individually and combination on PRSV in snake gourd plants of var. Kaumudi at 40, 50 and 60 DAS

Treatment	Fold increase in OD value over absolute control		
	40 DAS	50 DAS	60 DAS
B. velezensis + virus	0.45 ± 0.04[d]	2.26 ± 0.03[d]	2.46 ± 0.02[d]
AMF + virus	0.50 ± 0.02[c]	2.54 ± 0.02[c]	2.84 ± 0.04[c]
P. indica + virus	0.33 ± 0.02[e]	2.05 ± 0.02[e]	2.15 ± 0.01[e]
B. velezensis + AMF + *P. indica* + virus	0.82 ± 0.03[b]	3.05 ± 0.03[b]	3.06 ± 0.02[b]
Virus alone	2.50 ± 0.03[a]	3.65 ± 0.04[a]	4.33 ± 0.05[a]
SE (m)	0.02	0.01	0.02
C.D	0.05	0.05	0.05

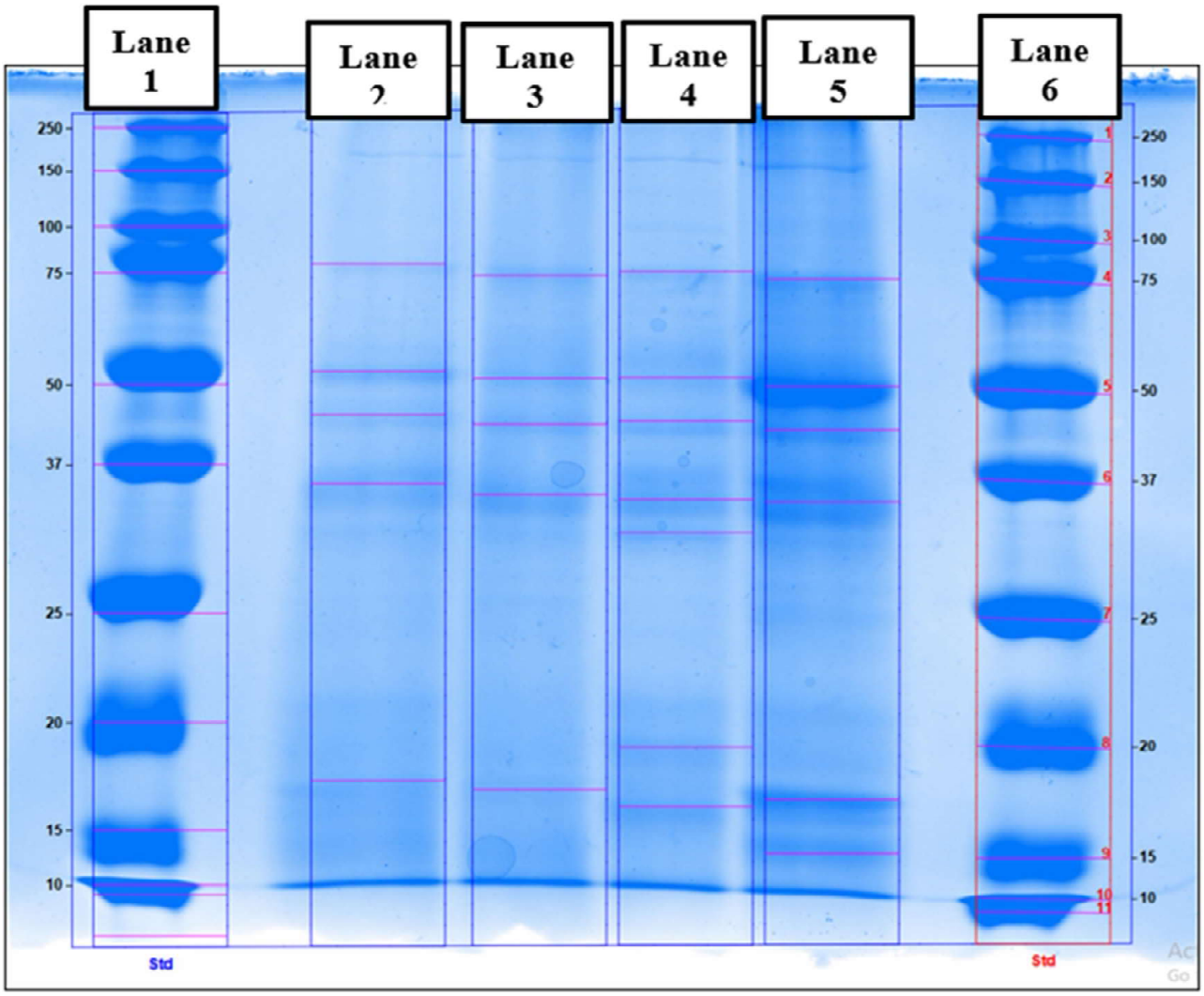
Values are mean of 3 replications ± standard deviation; SE: standard error; C.D: critical difference; DAS: days after sowing

cleaner SDS-PAGE profile with fewer viral protein bands, indicating more efficient protein turnover and reduced accumulation of harmful proteins. Such proteolytic activity supports plant defense by preventing excess viral protein buildup and maintaining cellular balance (Ghaffari, 2019).

Snake gourd plants pre colonized with AMF expressed the highest number of profiled proteins (seven proteins) having molecular weights of 78.42, 52.05, 44.64, 34.55, 31.57, 19.50 and 16.67 kDa, with a novel induced protein (31.57 kDa) when compared to control plants. This novel protein is likely a de novo defense molecule, potentially a pathogenesis related protein or a chaperone induced by the combined effects of AMF colonization and viral infection (Domingo et al., 2023).

Field studies

P. indica colonization resulted in the highest number of leaves at 30, 45 and 60 DAS (21.40, 32.60, and 41.20) followed by colonization with *B. velezensis* PCSE 10 (15.20, 27.20 and 36.80) and AMF (13.80, 24.60 and 31.20) (Table 4). The days taken for flowering decreased (36.00±1.581) in *P. indica* colonized plants followed by plants colonized with *B. velezensis* PCSE 10 (43.00) and AMF (49.40). Pre endophytic colonization with *P. indica* recorded the highest number of fruits, weight/fruit and yield (7.80, 0.91 kg and 7.04 kg/plant) followed by colonization with *B. velezensis* PCSE 10 (5.60, 0.75 kg and 4.15 kg/plant) and AMF (3.90, 0.73 kg and 2.77 kg/plant). Similarly, *P. indica* colonization improved growth and yield parameters in virus infected tomato (Sam, 2021) and papaya (Joy, 2024) under field conditions.



Lane 1: Biorad protein standard; Lane 2: *P. indica*+Virus; Lane 3: *B. velezensis* PCSE 10+Virus; Lane 4: AMF+Virus; Lane 5: Virus control; Lane 6: Biorad protein standard

Fig. 2 : Protein profile in response to pre-endophytic colonization in snake gourd leaves against snake gourd mosaic disease on sap transmission

Table 4 : Growth promotion of endophyte colonized snake gourd plants of var. Kaumudi under natural field conditions

Treatment	No. of leaves			No. of days taken for flowering	No. of fruits/ plant	Weight/ fruit (kg)	No. of fruits
	30 DAS	45 DAS	60 DAS				
P. indica	21.4±1.140[a]	32.6±2.074[a]	41.2±1.304[a]	36.0±1.581[a]	7.8±0.837[a]	0.907±0.071[a]	7.037±0.441[a]
B. velezensis PCSE 10	15.2±0.837[b]	27.2±0.837[b]	36.8±1.304[b]	43.0±1.581[b]	5.6±0.548b	0.749±0.134b	4.149±0.502b
AMF	13.8±0.837[c]	24.6±1.140[c]	31.2±1.304[c]	49.4±1.673[c]	3.9±0.447c	0.735±0.088b	2.766±0.172c
Control	11.6±1.140[d]	18.2±1.304[d]	25.4±3.209[d]	53.6±2.302[d]	3.5±0.837c	0.409±0.091c	1.497±0.067d
SE (m)	0.418	0.686	0.845	0.631	0.344	0.05	0.174
CD (0.05)	1.289	2.112	2.602	1.945	1.06	0.174	0.537

Values are mean of 3 replications ± standard deviation; SE: standard error; C.D: critical difference; DAS: days after sowing

CONCLUSION

The present study is the first report on successful colonisation of *P. indica* on snake gourd roots and its beneficial effect on reducing the severity of snake gourd mosaic disease by decreasing viral load of PRSV and CMV. The study identified the endophytism of *P. indica*, AMF and *B. velezensis* as a viable biocontrol strategy for managing snake gourd mosaic disease. Further research is warranted to elucidate the biochemical mechanisms involved in this host-pathogen interactions.

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