Beneficial root endophytic fungus *Piriformospora indica* inhibits the infection of *Blackeye cowpea mosaic virus* in yard long bean with enhanced growth promotion

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Abstract

Viral diseases are the major constraints in production and yield of yard long bean (vegetable cowpea), of which, *Blackeye cowpea mosaic virus* (BlCMV), a *Potyvirus* has been identified as a major threat. Management of diseases caused by plant viruses are very difficult but the use of beneficial root endophytes like *Piriformospora indica* is gaining importance in recent years for plant disease control. The present study was undertaken to utilize the potential of *P. indica* for the management of blackeye cowpea mosaic disease of yard long bean caused by BlCMV. The co-cultivation of *P. indica* with yard long bean and *Chenopodium amaranticolor,* the systemic and local lesion hosts of BlCMV respectively, was standardized. The seedlings of *Chenopodium* colonized with *P. indica* significantly reduced the number of local lesions produced by BlCMV over non-colonized plants. Yard long bean roots colonized with the endophytic fungus, *P. indica* significantly delayed the symptom development and also reduced vulnerability index to 70 per cent over the non-colonized plants with enhanced shoot and root biomass. This showed that *P. indica* could be used for managing viral diseases in crop plants with enhanced biomass to produce safe-to-eat farm produce.

Key words: Blackeye cowpea mosaic virus, *Piriformospora indica*, Vulnerability index, Yard long bean.

Introduction

Yard long bean is a protein enriched vegetable legume, cultivated in tropical and sub-tropical region. It is widely cultivated in South India and the climatic conditions favour year round production of yard long bean in Kerala (Kamala et al., 2014). The tender pods contain high protein (28%), iron, calcium, phosphorus, vitamins and dietary fibre (Singh et al., 2001). The major threats for cultivation of yard long bean are pests and diseases, of which, *Blackeye cowpea mosaic virus* and *Cowpea aphid-borne mosaic virus* are the major viruses affecting the crop (Frison et al., 1990).

In Kerala, the major virus infecting yard long beans is *Blackeye cowpea mosaic virus* (Krishnapriya, 2015). *Blackeye cowpea mosaic virus* (BlCMV) was initially identified in Florida by Anderson (1955). The incidence of viral disease under natural field condition is 66.6 per cent (Bashir et al., 2002) and the frequency of seed transmission is as high as 30.90 per cent (Zettler and Evans, 1972). Fungicides and antibiotics are the easiest and reliable means for the management of phytopathogenic fungi and bacteria but we lack potent viricides for the management of viral diseases. There are reports on beneficial endophytic fungi and bacteria mediated resistance against viral

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diseases in plants (Al-ani and Adhab, 2012; Lee et al., 2017; Lin et al., 2019).

Incidence of *Barley yellow dwarf virus* was lower in endophyte colonized meadow rye grass compared to non-colonized plants (Lehtonen et al., 2006). Cucumber roots colonized with arbuscular mycorrhizal fungus (AMF), *Glomus mosseae* and plant growth promoting fungus, *Fusarium equiseti* significantly reduced *Cucumber mosaic virus* (Elsharkawy et al., 2012). Squash plants colonized with *Beauveria bassiana* were effective against *Zucchini yellow mosaic virus* (Jaber and Salem, 2014). Muvea et al. (2018) reported that the endophytic fungus, *Hypocrea lixii* colonized plants inhibited the replications of *Iris yellow spot virus.* Lee and Ryu (2016) observed that the leaf colonizing bacteria, *Bacillus amyloliquefaciens* protected chilli against mechanically transmitted *Cucumber mosaic virus* and natural incidence of *Broadbean wilt virus* and *Pepper mottle virus* by enhancing salicylic acid and jasmonic acid defense signallings in plants. Leaf colonizing yeast *Pseudozyma churashimaensis* protected chilli plants against *Xanthomonas axonopodis* in addition to *Cucumber mosaic virus, Pepper mottle virus*, *Pepper mild mottle virus* and *Broadbean wilt virus* by inducing PR-proteins (Lee et al., 2017). *B. amyloliquefaciens*-primed plants suppressed *Tobacco streak virus* in cotton (Vinodkumar et al., 2018) and the bacterial endophyte secreted ten antimicrobial polypeptides along with pyrrole and deconic acid which impart antiviral property.

Piriformospora indica is a root colonizing beneficial basidiomycetous fungus which promotes plant growth with enhanced root and shoot biomass in both monocots and dicots; and confers tolerance / resistance to fungal, bacterial and viral diseases in addition to abiotic stress viz., drought, water stress, salinity, extreme temperature, heavy metals, oxidative stress (Oelmuller et al., 2009; Johnson et al., 2014; Gill et al., 2016). In addition to growth promotion, *P. indica*-colonized tomato plants suppressed the disease symptom expressed by *Pepino mosaic virus* and the virus concentration was decreased with increase in colonization (Fakhro et al., 2010). Wang et al. (2015) observed that *P. indica*-primed tomato plants induced resistance against *Tomato yellow leaf curl virus* in susceptible variety with enhanced growth. In this context, the present study was undertaken to utilize the potential of *P. indica* against BlCMV infection in yard long bean.

Material and methods

Maintenance of the virus

Blackeye cowpea mosaic virus was maintained in yard long bean variety Sharika (*Vigna unguiculata* var. *sesquipedalis* (L.) Verdcourt and the local lesion host, *Chenopodium amaranticolor* by sap transmission. Young yard long bean leaves showing typical symptoms like mosaic, leaf malformation and vein banding were collected from field and one g of leaf sample was homogenised in a pre-chilled mortar and pestle with 1.5 ml of 0.1 M sodium phosphate buffer (pH 7.0). The sap was filtered through muslin cloth and placed in ice box for immediate inoculation into selected cultivars. Primary two leaf stage of cowpea plants are susceptible to infection and 9 to 10 leaf stage of *C. amaranticolor* plants was selected for inoculation. *C. amaranticolor* was exposed to dark conditions prior to inoculation. Leaves were uniformly dusted with carborandum powder (600 mesh) and inoculation was done by dipping cotton in sap and gently rubbed on the dusted plant surface. After five minutes or prior to drying of sap, leaves were rinsed with distilled water using wash bottle.

Maintenance of the fungal root endophyte P. indica

The beneficial fungal root endophyte *P. indica* from Department of Plant Pathology, College of Agriculture, Vellayani, was maintained in Potato Dextrose Agar (PDA) medium and Potato Dextrose Broth (PDB). Fungal disc from actively growing margin of two week-old culture of *P. indica* was transferred to petri plates containing PDA and incubated in dark at room temperature. It was sub-

cultured once in fifteen days. *P. indica* inoculated medium was incubated at 27±3°C in dark.

Co-cultivation of P. indica with Chenopodium amaranticolor and yard long bean

C. amaranticolor and yard long bean were co-cultivated as per the protocol of Johnson et al. (2011; 2013). *P. indica* was inoculated into jam bottles and petri plates containing modified PNM medium, and incubated in dark for growth of the fungus (Johnson et al., 2011; 2013). Yard long bean and *Chenopodium* seeds were surface sterilized with 0.1 per cent mercuric chloride followed by three washings in sterile water, air dried and placed in two week old culture of *P. indica* in PNM. It was maintained in controlled condition for colonization.

The planting medium was prepared by mixing vermiculite-perlite of 3:1 ratio on volume basis and sterilised for one hour for three days. Harvesting of mycelia from PDB was done by filtration through muslin cloth, and mycelia were washed three times with sterile distilled water to make it free from adherent medium. It was weighed and one per cent of mycelium was added to vermiculite-perlite media (w/w). It was filled in pots and seeds of yard long bean and *Chenopodium* were planted for colonization.

Analysis of root colonization

Roots from co-cultivated plant were collected at weekly intervals to examine the fungal colonization. Roots were washed in tap water to free from potting media and cut into pieces of one cm. They were subjected to ten per cent potassium hydroxide at 65°C for 10 min, washed in distilled water, neutralized by treating with one per cent of HCl for five min and stained in lactophenol-tryphan blue for 10 min. Presence of mycelium, chlamydospores and colonization were examined under compound microscope.

P. indica-priming and challenge inoculation of BlCMV in C. amaranticolor and yard long bean

The procedure for co-cultivation of plants with *P. indica* and the virus transmission was the same as detailed above. BlCMV was challenge inoculated on *Chenopodium* and yard long bean, 24 h before and after root colonization with *P. indica* to evaluate its ability to reduce the symptoms after and before BlCMV infection. The experiments were carried out in completely randomized design. Root and shoot weight of yard long bean plant was measured. The per cent inhibition of local lesion over control in *C. amaranticolor* was calculated based on the formula

$$\text{Per cent inhibition} = \frac{C - T}{C} \times 100$$

C – number of lesions on control leaves
T – number of lesions on treated leaves

Based on the symptom expressed by BlCMV in the inoculated yard long bean, vulnerability index (V.I.) was calculated in accordance with the scale 0-5 developed by Bos (1982) as mentioned below:

0 - no symptom
1 - slight vein clearing, very little mottling of light and dark green colour in younger leaves – resistant (R)
2 - mottling of leaves with light and dark green colour – medium resistant (MR)
3 - blisters and raised surface on the leaves – medium susceptible (MS)
4 - distortion of leaves – susceptible (S)
5 - stunting of the plant with negligible or no flowering and fruiting – highly susceptible (HS)

Based on the above scale, rating was assessed and vulnerability index was calculated by using the equation,

$$VI = \frac{(0n_1 + 1n_2 + 2n_3 + 3n_4 + 4n_5)}{nt\ (nc-1)} \times 100$$

VI - Vulnerability Index
n0, n1….n5 - Number of plants in the category 0, 1, 2, 3, 4, 5
nt - Total number of plants
nc - Total number of categories

Results and discussion

Symptom development in Chenopodium and yard long bean

Blackeye cowpea mosaic virus was maintained in systemic host, yard long bean variety Sharika, by mechanical transmission using 0.1 M sodium phosphate buffer (pH 7.0). All the inoculated yard long bean seedlings expressed symptom on trifoliate leaves at 7th day after inoculation (DAI). Inoculated yard long bean plants initially produced vein clearing or vein banding symptoms on the emerged first trifoliate leaves. Vein clearing symptoms were also observed in seedling raised from seeds of virus infected plants. The typical symptoms included mosaic, leaf blistering, vein netting, reduction in leaf size and malformation. Severely infected plants were stunted with floral malformation and reduced pod length with few seeds (Plate 1). Symptoms produced on mechanically inoculated plants were

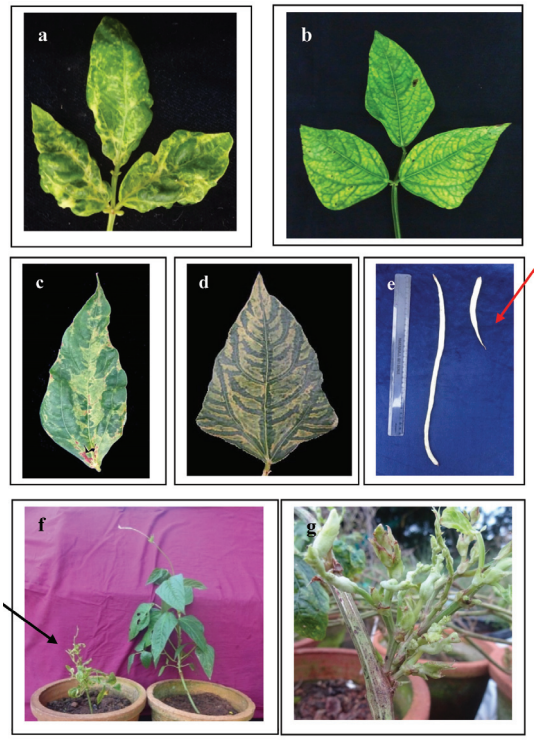

Plate 1. Common symptoms of BlCMV in yard long bean. (a) Leaf blistering; (b) vein netting; (c) mosaic; (d) Vein banding; (e) pod length reduction; (f) stunting; (g) flower malformation

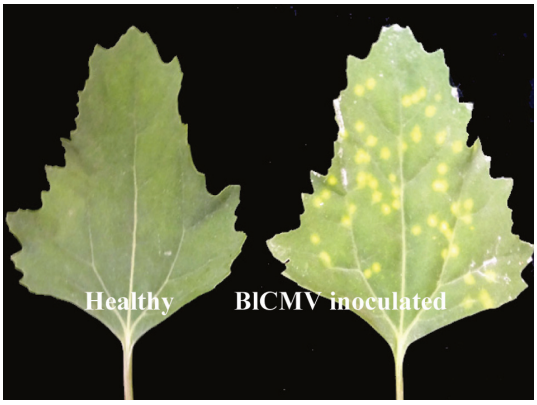

Plate 2. Local lesions symptom of BlCMV in *C. amaranticolor*

similar to those under natural field conditions. In *Chenopodium*, local lesions appeared as minute spots on fourth day and yellow chlorotic localized lesions of 2 to 3 mm in size were observed on 5th DAI (Plate 2). Similar symptoms of vein clearing, vein banding and mosaic symptoms were reported by Collins et al. (1985), in addition to blistering and malformation (Ouattara and Chambliss, 1991; Shilpashree, 2006; Alex, 2017). Symptoms such as reduced flowers and pods with malformed leaves (Krishnapriya, 2015); upward and downward leaf curling, crinkling and mottling (Dhanasekar and Reddy, 2015) were also reported, which were similar to the symptoms observed in the present study. In *Chenopodium*, yellow chlorotic local lesions appeared five days after BlCMV inoculation which later changed into necrotic lesions. Similar observation on chlorotic local lesions turning to necrotic was reported by Pavithra et al. (2015). BlCMV infection was studied in *Chenopodium* and the virus produced localized yellow chlorotic lesions (Saric, 1991; Radhika, 1999; Veena, 2007; Alex, 2017).

Colonization of P. indica on Chenopodium and yard long bean

The beneficial fungal root endophyte *P. indica* was maintained in potato dextrose agar (PDA) and potato dextrose broth (PDB) by continuous sub-culturing at every fifteen days intervals. The fungus attained 9 cm radial growth after nine days of

inoculation in petri plate containing PDA medium and it took twenty one days to form mycelial mat on 100 ml PDB in 250 ml conical flask (Plate 3).

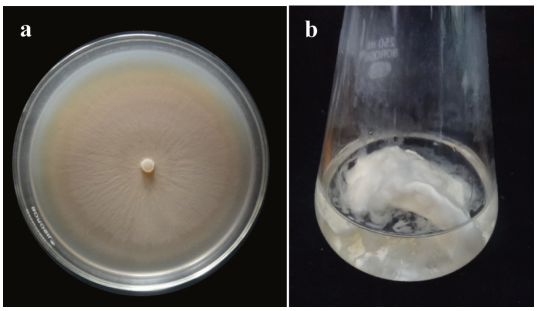

Plate 3. Maintenance of *P. indica* in potato dextrose medium (a) Radial growth of *P. indica* in potato dextrose agar on 10th day; (b) Growth of *P. indica* in PD broth on 15th day

Yard long bean seedlings in cotyledonary stage, raised directly from seeds were placed on two weeks old mycelia lawn of *P. indica* in jam bottles containing PNM medium. The mycelia were observed in roots after five days of co-cultivation and chlamydospores were produced on root surface

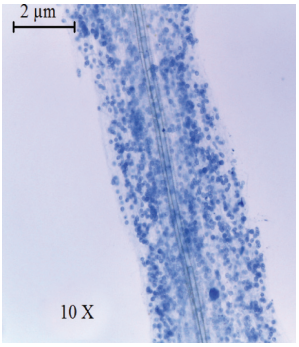

Plate 4. *In vitro* root colonization of *P. indica* in *C. amaranticolor*, three weeks after co-cultivation

on seventh day after co-cultivation (Plate 5). On 14th day, chlamydospores clumped together on the root and colonized within the roots.

In *Chenopodium*, viable seeds were directly placed on fully grown *P. indica* mycelial lawn of two week old culture in PNM medium. *Chenopodium* germinated in two weeks and colonization of the fungus was observed in roots at three weeks after co-cultivation (Plate 4). *In vitro* co-cultivation of *P. indica* with *Chenopodium* was difficult due to poor and non-uniform germination of the seeds.

Co-cultivation of yard long bean and *Chenopodium* with *P. indica* were also done in protrays containing vermiculite-perlite (3:1) medium mixed with 1 per cent mycelium of *P. indica*. Roots were observed for colonization in intervals. In yard long bean, the mycelia were seen within roots after five days and chlamydospores on the root surface after seven days of co-cultivation (Plate 5). Chlamydospores were seen inside the colonized roots on 10th day after co-cultivation. The colonization pattern was similar to previous method of colonization of yard long bean with *P. indica* under *in vitro* condition.

In *Chenopodium*, the mycelia were observed in roots within a week of co-cultivation. Chlamydospores were produced on root surface within two weeks (Plate 4) and in three weeks inside the roots. Thus, *P. indica* took more than 21 days for chlamydospore production in *Chenopodium* and 14 days in yard long bean roots under *in vitro* and *in vivo*.

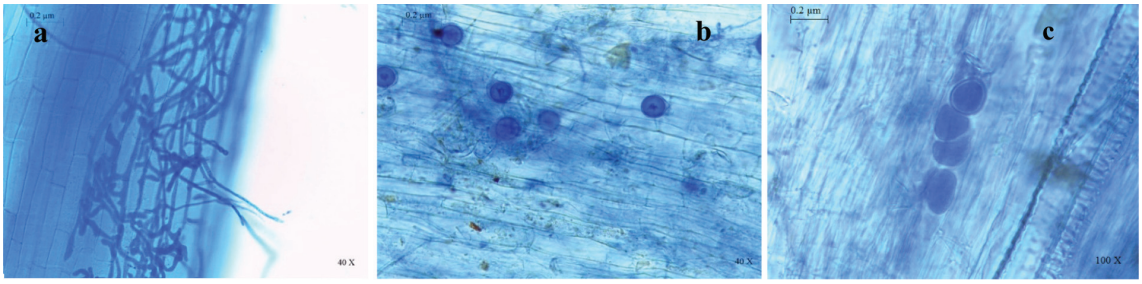

Plate 5. *P. indica* colonization in yard long bean. (a) mycelium in roots 5 DAC; (b) Chlamydospores in roots 7 DAC; (c) colonization in roots 14 DAC

Inhibition of BlCMV in P. indica-primed yard long bean with enhanced growth promotion

P. indica is a novel fungal root endophyte that enhances defense mechanism of plants against various biotic and abiotic stress and its effectiveness against BlCMV in *Chenopodium* was evaluated. *Chenopodium* plants co-cultivated with *P. indica* for three weeks and un-colonised healthy plants were challenge inoculated with BlCMV. The local lesion symptoms were expressed five days after inoculation in the un-colonised healthy plants inoculated with BlCMV but *P. indica* colonized plants significantly and remarkably inhibited the local lesions produced by BlCMV. The per cent inhibition of BlCMV local lesion in the *P. indica*-colonised plants ranged from 60 to 73 per cent with an average inhibition of 68 per cent over control (Table 1; Plate 6 and 7). Similarly in yard long beans, *P. indica*-colonisation resulted in significant and substantial inhibition of the cowpea blackeye mosaic disease compared to un-colonised yard long bean plants. Yard long bean is a systemic host of BlCMV and symptoms are expressed throughout the plant. The intensity of viral infection is determined by assessing the vulnerability index. The highest reduction of the disease was recorded in *P. indica*-colonized yard long bean plants prior to virus inoculation (71 % over control) followed by post-inoculated plants. Treatments that were applied prior to virus inoculation showed less vulnerability index to the viral infection than the post-inoculation (Table 2; Plate 8 and 9).

Table 1. Effect of *P. indica*-priming on BlCMV in *C. amaranticolor*

Treatment	Days for symptom appearance	Lesion size (mm)	Number of lesions	Per cent inhibition over control
P. indica alone	0	0	0	0.001
BlCMV alone	5	0.5	15.8 ± 0.86	0.00
P. indica-primed seedlings + BlCMV	5	0.5	4.8 ± 0.37	67.99±2.49
Control	0	0	0	0.001
SE (m) ±			1.4	3.73
CD (0.05)			0.46	1.24

Values are the mean of five replications ± standard deviation

Table 2. Effect of *P. indica*-priming in yard long bean against BlCMV

Treatment	Days for symptom appearance	Vulnerability index at 30 DAI	Per cent inhibition over control
P. indica alone	0	0	0.001
BlCMV alone	5	73.33	0.00
P. indica-primed seedlings + BlCMV	5	20.00	72.72
BlCMV + *P. indica*-primed seedlings	5	26.66	63.64
Control	0	0	0.00

Values are the mean of five replications

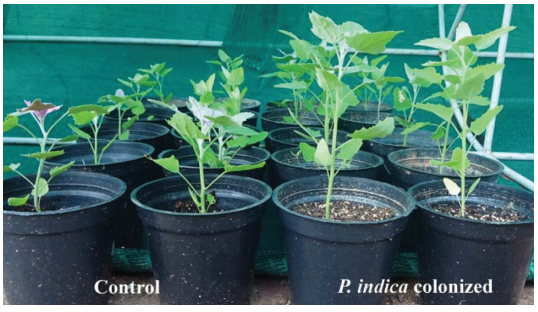

Plate 6. Effect of *P. indica*-priming of *C. amaranticolor* on BlCMV and growth promotion

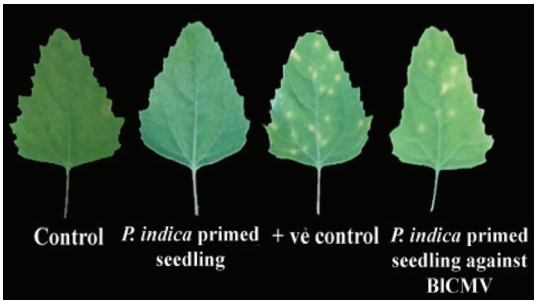

Plate 7. Effect of *P. indica*-primed *C. amaranticolor* to BlCMV

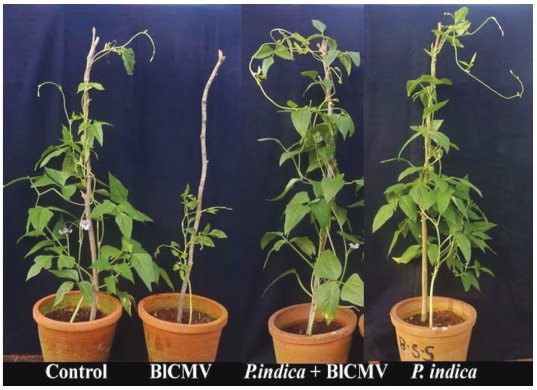

Plate 8. Effect of *P. indica*-priming of yard long bean plants on BlCMV

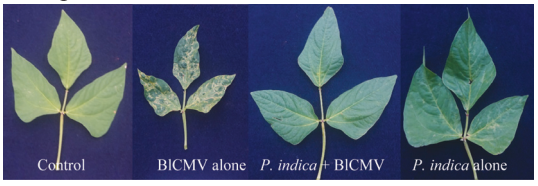

Plate 9. Effect of *P. indica*-priming of yard long bean plants against BlCMV

The results were in agreement with previous findings that the *P. indica*-colonized tomato plants could suppress more than 75 per cent of symptoms expressed by *Tomato yellow leaf curl virus* and enhance PAL activity with induction of PR-protein (Wang et al., 2015). Fakhro et al. (2010) reported that *P. indica*-colonised plants were able to reduce *Pepino mosaic virus* concentration in tomato under high light intensity. Khalid et al. (2019) stated that *P. indica* enhanced tolerance to plants by inducing antioxidants and induced stress regulated genes. Al-ani and Adhab (2012) found that beneficial biotic agents applied to roots indirectly mediated resistance against plant virus by producing compounds possessing antiviral property. Anthurium plants colonized with *P. indica* stimulated higher activities of stress related enzymes, jasmonic acids and genes of jasmonic acid mRNAs, which in turn were responsible for resistance to plants against biotic stress (Lin et al., 2019).

Root and shoot biomass of the treated plants were assessed and the *P. indica*-primed plants inoculated

Table 3. Effect of *P. indica*-priming on root and shoot biomass of yard long bean

Treatments	Root biomass (g)	Shoot biomass (g)
Control	18.31 ± 2.08	124.37 ± 2.01
BlCMV alone	4.65 ± 1.98	13.65 ± 2.43
P. indica-primed seedlings + BlCMV	22.70 ± 2.40	144.89 ± 3.28
P. indica alone	28.45 ± 5.80	128.68 ± 14.48
SE (m) ±	3.45	7.59
CD (0.05)	11.44	25.14

Values are the mean of three replications ± standard deviation

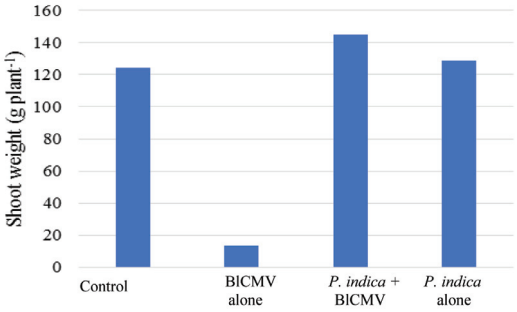

Figure 1. Effect of *P. indica* on shoot biomass of yard long bean

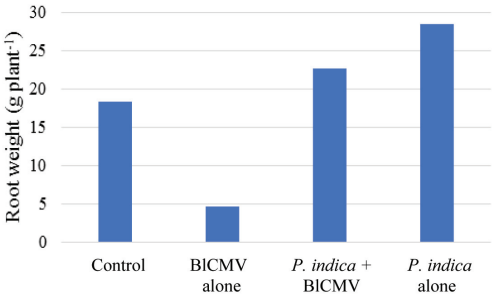

Figure 2. Effect of *P. indica* on root biomass of yard long bean

with the virus yielded higher root and shoot biomass than healthy plants (Table 3; Fig 1). In post-inoculation studies, biomass of the treated plants was less compared to the pre-inoculated plants and it was highest in *P. indica*-colonized plants (Table 3; Fig 2). *P. indica*-primed virus inoculated plants had significantly higher biomass. *P. indica* directly acted on plant hormone signalling pathway to induce IAA, cytokinin and other growth regulators (Johnson et al., 2014). It enhanced root proliferation

resulting in better nutrient uptake, increased crop growth and productivity (Lee et al., 2011; Gill et al., 2016; Desisa, 2017). Thus, the present study revealed that BlCMV could be managed by pre- and post- treatment of yard long bean / vegetable cowpea with root colonization of *P. indica*. The combined effect of *P. indica* with other beneficial root or shoot endophytes for management of BlCMV in yard long bean / vegetable cowpea has to be explored.

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