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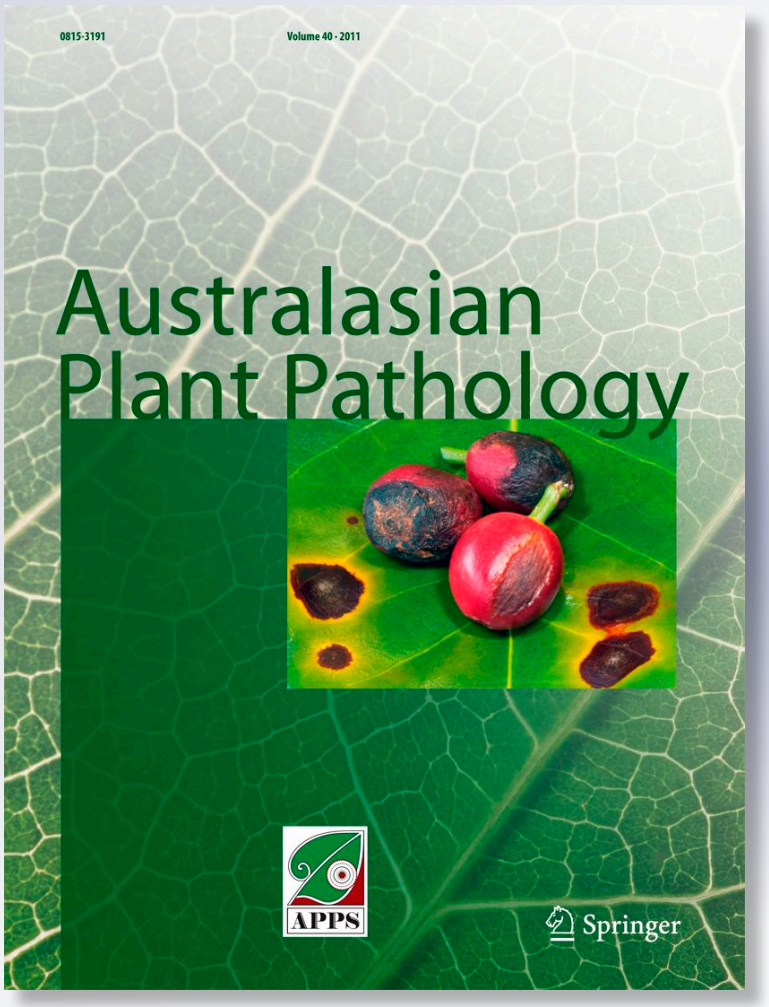

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Evaluation of vermicompost doses for management of root-rot disease complex in *Coleus forskohlii* under organic field conditions

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Abstract Organic field experiments were conducted (2007–09) for the medicinal plant *Coleus forskohlii* with different doses of vermicompost to minimize the root-rot disease incidence and increase the growth and yield. The use of vermicompost significantly increased the growth parameters in general but the maximum plant height, plant spread and number of branches increased with the application of 5 t ha^{-1} of vermicompost. Root-rot/wilt, a complex disease of *C. forskohlii* involving *Fusarium chlamydosporum* and *Ralstonia solanacearum*, was significantly ($P<0.05$) reduced in terms of percent wilt incidence (PWI) and severity of root-rot as measured by percent disease index (PDI) with different doses of vermicompost. The highest levels of biomass (root and shoot; 48 % and 71 %, respectively) and forskolin yield (46 %) and disease suppression (PWI and PDI; 73 % and 82 %, respectively) were found at the top level of vermicompost (5 tha^{-1}). Nutrient (NPK) uptake was significantly elevated in plots supplemented with vermicompost compared to control plots.

Keywords *C. forskohlii* · Vermicompost · Disease management · Organic farming

Introduction

Coleus forskohlii (family Lamiaceae) grows perennially in tropical and subtropical regions of India, Pakistan, Sri Lanka, East Africa and Brazil. Its roots are the source of a labdane diterpene compound called forskolin having a unique property to stimulate adenylate cyclase. Forskolin is also a potent vasodilatory, anti-hypertensive and inotropic agent (Seamon 1984). The crop has a great potential due to the expected increase in demand for forskolin, which is widely used in glaucoma, cardiac problems and also in the treatment of certain types of cancers (Shah et al. 1980). Its ethnomedicinal uses for relief of cough, eczema, skin infections, tumors and boils have been recorded (De Souza et al. 1986). Because of continuous collection of roots from wild sources, this plant has been included in the list of endangered species (Boby and Bagyaraj 2003). Recently, its cultivation has picked up as a crop with annual production of about 100 tons from 700 ha in India (Shivkumar et al. 2006).

C. forskohlii is susceptible to many diseases of which root-rot and wilt is the most important, causing serious losses. The pathogen causing the disease has been identified as *F. chlamydosporum* (Singh et al. 2009a). Soil borne diseases are complex because of the array of organisms associated with it. *Ralstonia solanacearum* has also been reported to cause vascular wilt in *C. forskohlii* (Coelho and Assis 2002).

Control of phytopathogenic organisms typically relies on use of synthetic chemicals (Elad et al. 2007). Their widespread use is implicated in the development of pathogen resistance to these pesticides (Elad et al. 1992), thereby threatening the stability of crop production. Elevated concentrations can cause reduced soil biological activity and subsequent loss of fertility in agricultural lands (Zwieten et al. 2007). Therefore, alternatives to these are extensively being explored to avoid the use of synthetic fungicides in order to sustainably produce safe agro-products.

Vermicomposts, or earthworm-processed organic wastes are finely divided peat-like materials with high porosity, aeration, drainage, and water-holding capacity (Edwards and Burrows 1988) containing substances that stimulate and regulate plant growth (Tomati et al. 1988). As an organic fertilizer,

R. Singh · S. K. Soni · A. Awasthi · A. Kalra (✉)
Department of Microbial Technology, Central Institute of Medicinal and Aromatic Plants (CSIR - CIMAP), P.O. CIMAP, Lucknow 226015, India
e-mail: alok.kalra@yahoo.com

vermicompost from wastes of medicinal and aromatic crops has been shown to improve soil nutrient status and overall health (Anwar et al. 2005). Vermicompost has also been found effective in suppressing root and soil-borne pathogens (Rodríguez-Navarro et al. 2000; Schönfeld et al. 2003; Yasir et al. 2009).

Vermicompost produced from distillation waste of industrial aromatic plants is inhibitory to phytopathogens such as *Fusarium*, *Ralstonia* and nematodes (Singh et al. 2009b; Pandey et al. 2011). The main objective of the present study was to develop successful organic cultivation practices in *C. forskohlii* by minimizing disease losses through field evaluation of organic fertilizer (vermicompost) for managing root-rot/wilt and improving root yields of *C. forskohlii* under organic field conditions.

Material and methods

Isolation of fungal and bacterial pathogen from infected plants

Both of the pathogens [*F. chlamydosporum* (Wollenweber & Reinking) and *R. solanacearum* (Smith)] were isolated from infected plants exhibiting typical root-rot and wilt symptoms grown at the Central Institute of Medicinal and Aromatic Plants (CIMAP) experimental farm at Bangalore, India. The identity of the fungal pathogen (*F. chlamydosporum*) was confirmed by Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi, India (Boby and Bagyaraj 2003) whereas the bacterial pathogen *R. solanacearum* was identified (white fluidal irregular colonies with pink centre) following the methods adopted by Kelman (1954) and Vanitha et al. (2009). The virulent *R. solanaceraum* (isolate CIMAP-R7) and *F. chlamydosporum* (isolate CIMAP-F6) are being maintained at −80°C with 50 % glycerol in the culture collection of CIMAP.

Preparation of *Coleus* cuttings

During both the years (2007–09) the *Coleus* cuttings were taken from a 5 months old healthy crop and raised in 15× 6 cm polyethylene bags containing 200 g of potting mixture (soil: sand; 1:1 v/v). Terminal parts of healthy stem were used for the preparation of cuttings. Planted cuttings were placed under 60 % shade net for 50 days for rooting. The plants were kept for 5–7 days in open condition to harden before transplanting to the field.

Production of quality vermicompost

CIMAP has developed and patented a technology for production of quality vermicompost utilizing distillation waste of aromatic oil crops (Kalra et al. 2002; Kalra et al. 2010; Singh et al. 2012). During both the experiments, vermicompost was produced from distillation waste (spent plant materials) of lemongrass (*Cymbopogon flexuosus*) and citronella (*Cymbopogon winterianus*) at the vermicompost production unit of the certified organic farm for a period of 90 days using an epigeic species of earthworm (adult clitellate *Eisenia foetida*). The vermicompost was stored in the shade in air tight polyethylene bags to retain the moisture content prior to application in organic plots. Samples were collected from sieved vermicompost (about 50 g) each year and analysed for NPK as depicted in Jackson (1973) and micronutrients (Zn, Fe, Mn, Cu) using DTPA method (Lindsay and Norvell 1978). The vermicompost contained essential macronutrients including 1.0 % N, 0.77 % P, 0.57 % K and micronutrient Zn, Fe, Mn and Fe (70 mg kg^{-1}, 225 mg kg^{-1}, 185 mg kg^{-1} and 42 mg kg^{-1}, respectively) on a dry weight basis. Culturable bacterial, fungal and actinomycetes population assessed in vermicompost (g^{-1}) were 3.4×10^{7}, 3.5×10^{5} and 2.1×10^{6}, respectively.

Field experimental design and transplanting

The experiment was conducted in a certified (ECOCERT) organic farm at the Central Institute of Medicinal and Aromatic plants, Research Center, Bangalore (India), located at 12° 58′ N 77°35′ E and 930 m above mean sea level. The soil of the experimental field was a red sandy loam with pH 6.2, 115 kg ha^{-1} available N, 10.1 kg ha^{-1} available P, 133.2 kg ha^{-1} available K and 0.40 % organic carbon. The treatments were imposed in plots (1.8×3.6 m) with 5 replications arranged in randomized block design (RBD) in naturally-infected soil. The rooted cuttings (50-day-old) of *C. forskohlii* were transplanted (28.12.2007) on ridges at a spacing of 60×45 cm (row to row 60 cm and plant to plant 45 cm) into holes having a depth of 10–12 cm and dia of 8–10 cm by placing the rooted cuttings with intact ball of earth (soil mixture) without polythene covers. There were four rows in each plot. Each plot was separated with 50 cm flat ridges (guard row) to mitigate the effect of adjoining plots. Each year, initial soil samples (200 g) were collected with the help of soil auger (0–15 cm) from five points of each replicated treatments. The soil samples were pooled, mixed and a subsample was used to record the pH, available N, P, K, percent organic carbon and initial population of pathogenic organism (*F. chlamydosporum* and *R. solanacearum*) prior to transplanting and application of vermicompost. Soil samples were passed through a sieve (<2 mm) and analysed for pH (soil: water 1:2.5), NPK following standard procedures (Jackson 1973). Soil organic carbon was analyzed following the method of Walkley and Black (1934). The soil had uniform infestation of root rot complex pathogens (*F. chlamydosporum* 3.0×10^{4} CFU g^{-1} soil and *R.*

solanacearum 3.5×10^6 CFU g^{-1} soil) throughout the field. Each bed contained 32 plants (20 border and 12 net plants) . Net plants were considered as non-peripheral plants (i.e. exludes border plants). Five net plants were randomly tagged (to avoid border effects) from each bed for taking growth observations.

There were six treatments: vermicompost at 1, 2, 3, 4, 5 t ha^{-1} and control (soil only). At the end of the first trial, the population of pathogens were more or less similar (*F. chlamydosporum* 3.32×10^4 CFU g^{-1} soil and *R. solanacearum* 3.61×10^6 CFU g^{-1} soil) in control plots, however, it ranged from 1.2 to 2.4×10^4 CFU g^{-1} and 2.2 to 3.6×10^6 CFU g^{-1} of soil in the case of *Fusarium* and *Ralstonia* respectively, with different doses of vermicompost. Re-transplanting (25.12.2008) of the *C. forskohlii* rooted cuttings was done in same manner (i.e. experimental design and rate of vermicompost application remained the same for same plots). Plants were grown for 150 days during both the trials.

Plant growth observations and wilt in field conditions

Plant parameters including plant height (measured from soil surface to the growing tip of the plant), number of branches and plant spread were recorded at the time of harvesting. Percent wilt incidence (PWI) (percentage of plants with yellowing and drooping of leaves and browning of vascular tissues of stem) was assessed in the field before harvesting the plants.

Harvesting

Harvesting was done 150 days after transplanting during both years of experimentation. Plants were manually uprooted with proper care without damaging the tubers. Fresh and dry root and shoot weights were recorded. 200 g samples of fresh shoot and root from each bed were dried in a hot air oven at 80°C for 24 h for determining the moisture content and nutrient uptake. From the moisture content and biomass yields, dry matter yields were calculated. 100 g fresh roots were chopped into 1 to 2 cm pieces and air dried by keeping the chopped roots on perforated mesh. The perforated mesh containing chopped roots were kept for 5–7 days on drying shelves (grid like structure made up of fine iron rods from which the air could pass) in the shade where the natural air passes from all-around the shelves. The powdered roots were used to determine the forskolin content. At the time of harvesting, rhizospheric soil samples were collected in the same fashion (as in initial soil sampling) to determine population of *Fusarium* and *Ralstonia* from each bed. Root-tubers from net plants were randomly selected from each plot for taking observations related to root-rot severity. Fresh and dry shoot-root yield from net plants of each bed was recorded whereas the data presented in Fig. 2 is on dry basis. Nutrient uptake in roots and shoots was determined (Singh et al. 2009a) based on NPK concentration (Jackson 1973) in dry matter samples and dry matter yields.

Percent disease index (PDI)

Disease severity was measured during both the trials on a 0–4 scale of Kesavan and Chowdhary (1977) where 0 = healthy roots (no symptoms), 1=1–25 %, 2=26–50 %, 3=51–75 % and 4=>75 % root tubers affected by rot (blackening, oozing and putrefaction of roots and plant death). Based on the scoring on root rot severity in all the three replicates of each treatment, the percentage disease index (PDI) was calculated as follows:

$$\mathrm{PDI} = (\text{Sum of numerical grading recorded}/\text{Number of roots observed} \times \text{highest numerical rating}) \times 100$$

Forskolin estimation

Forskolin in the *C. forkohlii* roots was estimated by High Performance Liquid Chromatography (HPLC). Powdered dried roots (1 g) were weighed and transferred into a conical flask (25 ml) to which 10 ml of acetonitrile was added. The samples were sonicated 4 times for 15 min. The combined extractions were concentrated and diluted to the final volume with 10 ml of acetonitrile. Before injection, each sample was filtered through 0.45 μm nylon filter membrane. Twenty microlitre of this was injected into a Shimadzu LC10ATVP HPLC. The column used was Phenomenex Luna 5 μm, C 18 (2) 100 A^0; 250 mm×4.6 ID, oven temperature 30°C, mobile phase acetonitrile: water with flow rate of 1 ml / minute. Standards preparation, calibration and gradient elution was done as per the reported method (Schaneberg and Khan 2003). Theoretical forskolin yield was calculated based on percent forskolin content and dry root yield but percent forskolin content is not presented in this paper.

Microbial population estimation

The density of pathogens population (*Fusarium* and *Ralstonia*) was estimated by using PCNB peptone agar (Nash and Snyder 1962) and 2,3,5 triphenyl tetrazolium chloride (TTC/TZC) semi-selective medium (Kelman 1954) respectively. Population of *Fusarium* obtained on PCNB peptone agar was then screened based on colony morphology, conidia and conidiophores structures of *F. chlamydosporum*. The isolates (*F. chlamydosporum*) from naturally infected soil produced a typical pinkish-red colony and abundant aerial mycelium on potato dextrose agar (PDA) medium. The morphology of conidia and

Table 1 Regression coefficient (b) and correlation coefficient (r) values for various traits in *Coleus forskohlii*

Various observation in *C. forskohlii*	Regression coefficient (b)	Correlation Coefficient(r)
Plant height (cm)	2.000	0.980
Plant spread (cm)	1.057	0.946
Number of branches	1.171	0.946
Dry shoot yield (t ha^{-1})	0.125	0.903
Shade dry root yield (t ha^{-1})	0.035	0.903
Forskolin yield (kg ha^{-1})	0.201	0.875
Percent disease index (PDI)	−6.096	−0.952
Percent wilt index (PWI)	−4.828	−0.915
Total N uptake (kg ha^{-1})	1.204	0.780
Total P uptake (kg ha^{-1})	0.283	0.770
Total K uptake (kg ha^{-1})	2.601	0.956
Fusarium×10^3 CFU g^{-1} soil	−0.31	−0.971
Ralstonia×10^6 CFU g^{-1} soil	−0.329	−0.928

Growth, biomass yield, disease incidence, nutrient uptake and pathogens population parameters are regressed against vermicompost doses (1, 2, 3, 4 and 5 t ha^{-1})

conidiophores were studied under a light microscope. Abundant spindle to club-shaped microconidia were borne on polyphialidic conidiophores. Macroconidia, which were typically sickle-shaped and with a foot-shaped basal cell, were developed on polyphialides from branched conidiophores. Chlamydospores were globose and rough-walled and sparsely formed at the end of short lateral hyphal branches on PDA medium after long-term incubation. These morphological characteristics of the isolates corresponded to the reference isolate of *F. chlamydosporum* (IFO-31096), and the description of the species by Booth (1971). Vermicompost samples were plated in triplicate using serial dilution technique on nutrient agar, rose bengal chloramphenicol agar and Ken-Knight agar media for counting the total number of aerobic bacteria, total fungi and actinomycetes, respectively (Collin and Lyne 1985; Allen 1959).

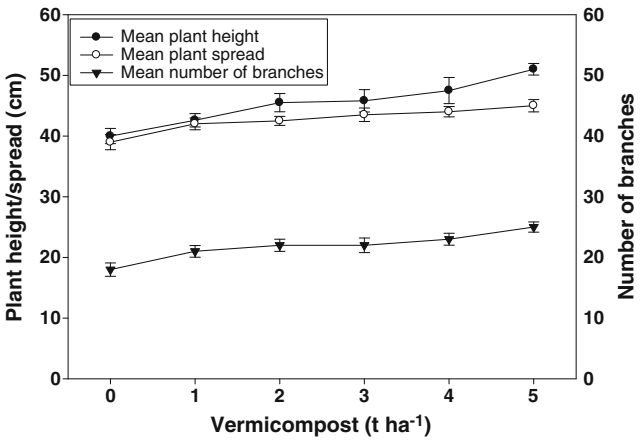

Fig. 1 Effect of graded dose of vermicompost on mean growth characteristics of *C. forskohlii*, Error bars are presented as standard error of mean (SEM)

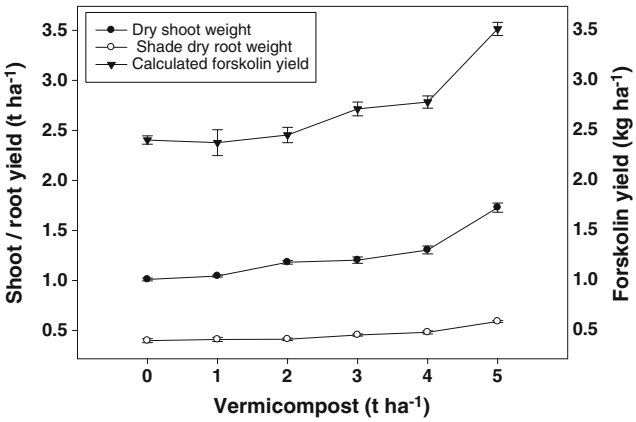

Fig. 2 Effect of graded dose of vermicompost on mean yields of *C. forskohlii*, Error bars are presented as standard error of mean (SEM)

Statistical analysis

Arcsine transformed values were used for PDI and PWI. Since the experimental data of two trials had a similar variance value therefore the data of both trials were pooled for further analysis. Linear regression was applied with the help of software Sigma plot 11 for all the parameters to get the regression coefficient (b) and correlation coefficient (r). Also, standard error of mean was computed with the help of Sigma Plot 11. Significant differences among treatments were based on the *t*-test at 95 % confidence level ($P \leq$

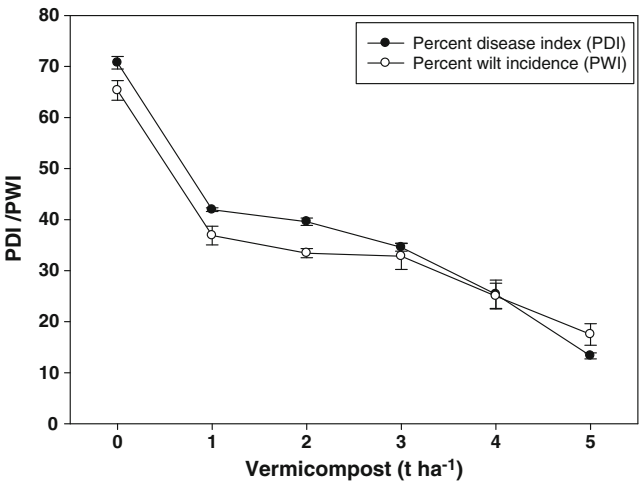

Fig. 3 Effect of graded dose of vermicompost on mean percent disease index (PDI) and percent wilt incidence (PWI) characteristics of *C. forskohlii*, Error bars are presented as standard error of mean (SEM)

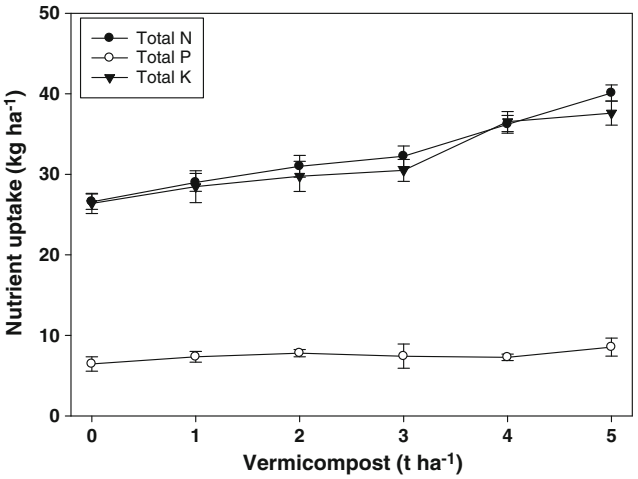

Fig. 4 Effect of graded dose of vermicompost on mean nutrient uptake of *C. forskohlii*, Error bars are presented as standard error of mean (SEM)

0.05). Results and discussion are based on the combined mean data of the two trials.

Result

Correlation analyses showed that vermicompost dose had a statistically significant relationship with plant height, plant spread and number of branches (Table 1). Plant height had a significant positive correlation with dose of vermicompost, with plants in 5 tha^{-1} treatment being 28 % taller than those without vermicompost. Plant spread and number of branches had also significant positive correlation with all the doses of vermicompost, however, highest plant spread (15 %) and number of branches (39 %) was observed in treatments receiving 5 tha^{-1} of vermicompost (Fig. 1) ($P \leq 0.05$).

A significant positive dose response effect of vermicompost was observed with reference to shoot, root and forskolin yields (b=0.125, 0.035 and 0.201 respectively) of *C. forskohlii* and also it was directly correlated (r=0.903, 0.903 and 0.875, respectively) with consecutive increase of vermicompost doses (Table 1). The highest root and shoot yields were observed with the upper limit dose of vermicompost (5 tha^{-1}), an increase of 48 and 71 %, respectively over control ($P \leq 0.05$). The forskolin concentration (0.61± 0.01 %) was, however, not affected by any of the doses of vermicompost. As a result of higher root yields, the total forskolin yield (calculated) was significantly higher in treatment with 5 tha^{-1} vermicompost (46 %) compared to non vermicompost treated plants (Fig. 2) ($P \leq 0.05$).

Regression analysis for arcsine transformed data of PWI and PDI was performed and that clearly exhibited a negative effect (b=−6.096 and −4.828, respectively) with increase in dose of vermicompost. However, the correlation coefficient (r) for PWI (−0.915) and PDI (−0.952) showed a strong negative correlation reflects with increase of vermicompost doses decrease of PWI and PDI observed (Table 1). All the doses of vermicompost (1 to 5 tha^{-1}) significantly reduced (44–73 %) the percent wilt incidence compared to control but the maximum reduction was observed with 5 tha^{-1} vermicompost (73 %) (Fig. 3). Apart from reducing the disease incidence, treatments with different doses of vermicompost also reduced significantly the severity of the disease as indicated by percent disease index; a reduction of 41–82 % compared to control (Fig. 3). The highest reduction (82 %) in disease index was observed in plants treated with 5 tha^{-1} vermicompost (Fig. 3).

It was found that total uptake of NPK was positively correlated (r=0.78, 0.77 and 0.96, respectively) with increase of vermicompost doses (Table 1). Total NPK uptake (shoot/root) improved significantly by the application of vermicompost. Plants receiving 5 tha^{-1} vermicompost

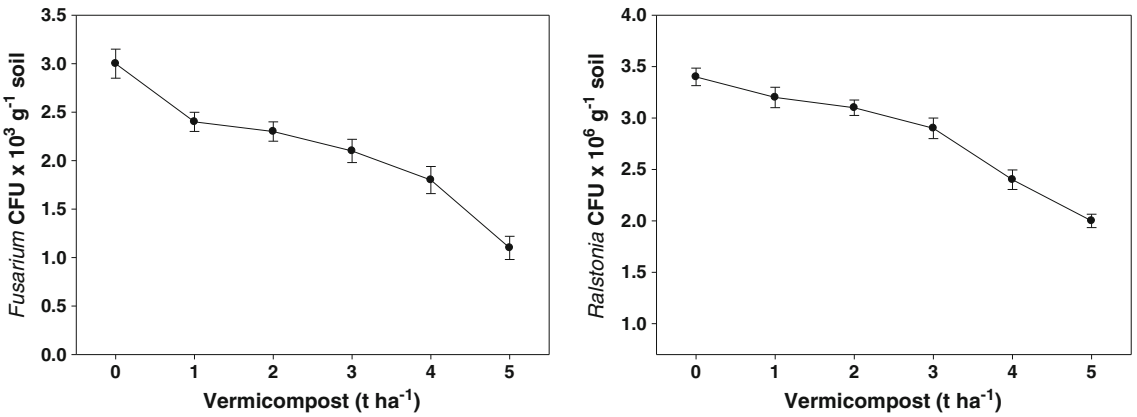

Fig. 5 Effect of graded dose of vermicompost on population of pathogenic organism in the rhizosphere of *C. forskohlii*, Error bars are presented as standard error of mean (SEM)

exhibited improved uptake of NPK; an increase of 32, 33 and 33 %, respectively (Fig. 4).

The population of *Fusarium* and *Ralstonia* prior to transplanting was 6×10^4 and 3.4×10^6 respectively. The population of pathogenic organisms *Ralstonia* and *Fusarium* associated with root rot/wilt showed a negative correlation ($r=-0.971$ and -0.928, respectively) with increase in vermicompost doses (Table 1). All the doses of vermicompost reduced the population of *Fusarium* significantly ($P<0.05$) by 40–67 % but the highest reductions war observed in plots with 5 tha^{-1} vermicompost (67 %). *Ralstonia* population also reduced significantly with various doses of vermicompost; however, the maximum reduction (41 %) was observed with highest level of vermicompost (Fig. 5).

Discussion

Findings of the present study are the similar to the previous studies showing improved plant growth response with the application of vermicompost in other crops such as tomato (Atiyeh et al. 2002). Vermicompost has a large surface area, providing strong absorption capability and retention of nutrients and contain nutrients (Shi-wei and Fu-zhen 1991) in forms that are readily taken up by plants such as nitrates, exchangeable P, and soluble K, Ca, and Mg (Orozco et al. 1996). Vermicompost may influence aboveground ecosystems by contributing to the plant nutrition, plant health, soil structure, and soil fertility in soil (O'Donnell et al. 2001). Physical and chemical properties of vermicompost may stimulate plant growth but there is also the possibility that indirect effects via the inhibition of plant pathogen infection may result in improved plant growth (Szczech 1999). Its richness in beneficial microorganisms (Atiyeh et al. 2000) affecting rhizosphere microflora (de Brito Alvarez et al. 1995), nitrate uptake kinetics (Dell' Agnola and Nardi 1987) and presence of plant growth regulators (Tomati et al. 1988) might override pure nutrient effects. Vermicompost, known for disease suppression (Schönfeld et al. 2003; Yasir et al. 2009), was successful in reducing the incidence and severity of the disease. Further, increase in vermicompost dose showed clearly a decrease in PWI and PDI during the present study. This becomes important as vermicompost apart from being a rich source of plant nutrients could suppress the pathogens and therefore becomes an important input for organic farming. Vermicompost as soil amendments, particularly in organically managed soils, can positively modify microbial-community composition of soil and would enhance the competition and/or antagonism among microbe leading to decrease in plant pathogens activity (Hoitink and Boehm 1999). Suppressive activities of compost against various pathogens are considered to be due to the antagonism by beneficial microflora (Hoitink et al. 1997; Keener et al. 2000) or due to the production and release of allelochemicals (Bailey and Lazarovits 2003). Apart from disease control, the higher yields could also be attributed to increased availability of nutrients because of application of vermicompost, as was also clearly reflected in the higher nutrient uptake by the plants grown with vermicompost (5 t ha^{-1}). Besides vermicompost being rich source of micronutrients may also promote the plant growth and yields. The present study clearly indicated that at a level up to 5 t ha^{-1} there was no evidence of adverse effect of adding too much vermicompost.

The results of the current experiments show that vermicompost from distillation waste of aromatic crops as a potent organic fertilizer has stimulatory effect on growth and biomass allocation of *C. forskohlii* as it can modify/improve the microbial communities by antagonising the pathogens. On the other hand it has considerable potential for reduction in root-rot and wilt complex of *C. forskohlii*. Application of vermicompost needs to be widely practiced owing to its better biological and nutritional properties for organic farming compared to general compost. This management approach will be particularly useful under organic farm conditions or especially in medicinal plants where the use of chemicals is restricted because of health and residue consideration. Recent study also suggests that vermicompost produced from the distillation of aromatic crops can serve as better substrate for multiplication and survival of beneficial microbes (Kalra et al. 2010; Singh et al. 2012).

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