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Review Article

Vermicompost and vermiwash add beneficial micro flora that enhance soil quality and sustain crop growth

Murali Gopal, Alka Gupta and George V. Thomas

Central Plantation Crops Research Institute, Kasaragod – 671 124, Kerala, India
Corresponding Author: mgcpcri@yahoo.co.in

ABSTRACT

Recycling agricultural and horticultural crop residues to vermicompost and vermiwash provide a good option for small and marginal farmers to produce solid and liquid organic manure locally for use in their farms. Vermicompost and vermiwash are well known to improve soil health and fertility as they add major and micro nutrients, organic matter, and plant growth promoting substances besides improving the soil structure. However, not much known but equally critical is the ability of vermicompost and vermiwash to add substantial load of plant-beneficial microbial communities to the soil that play an important role in augmenting the soil microbial diversity, nutrient mineralization, pathogen/nematode suppression and organic matter degradation resulting in better soil resistance and resilience. More importantly, addition of the vermicompost and vermiwash provides much needed food for the microorganisms in soil thereby stimulating their activities that is necessary for a soil to be fit for supporting good crop growth. In this article, we highlight the evidence gathered through conventional and molecular protocols on the important perspective of coconut leaf vermicompost and vermiwash being the foci of dissemination of plant-beneficial microorganisms and their ability to sustain the crop production capacities of the soils.

Keywords: coconut, vermicompost, vermiwash, microflora, recycling wastes

INTRODUCTION

It has been reported that the wastes generated annually in India from field and horticultural crop residues, agro-processing residues, animal/poultry excreta and livestock/fisheries wastes amount to about 1566 million tonnes. Producing quality manure through composting and vermicomposting technologies with stringent standards as well as generating bio-energy through anaerobic microbial fermentation and pyrolysis has been suggested for effective utilization of these voluminous agro-wastes (NAAS, 2010).

Recycling wastes to vermicompost and vermiwash

Vermiculture biotechnology for producing vermicompost using earthworms from the agro-wastes is a simple and useful technology and has been proposed as one of the front runner technologies for realizing the second green revolution and sustainable agriculture (Chaudhary *et al*., 2004; Sinha *et al*., 2010). Vermicomposting is the bio-oxidation and stabilization of organic matter involving the joint action of earthworms and microorganisms, thereby turning wastes into a valuable soil amendment called vermicompost (Dominguez *et al*., 2010). The earthworm species (or composting worms) most often used are Tiger Worms or Red worms (*Eisenia foetida*) or Red Wrigglers (*Lumbricus rubellus*) or African night crawlers (*Eudrilus eugeniae*) and Indian blue worm (*Perionyx excavatus*). These species are rarely found in soil and are adapted to the special conditions in rotting vegetation and compost and manure piles (Kale, 1998). A wide range of agri-horti wastes have been used as substrates for production of vermicompost using one or the other above mentioned earthworms (Edwards, 1998; Jeyabal & Kuppuswamy, 2001; Garg *et al.,* 2006; Barik *et al.,* 2010).

Vermiwash is a liquid organic fertilizer generated as a spin off technology from vermicompost production (Ismail, 1997). It can be done basically by two methods. One involves soaking soil + cow dung + earthworms substrate in excess water in plastic tub and siphoning the wash periodically from the bottom of the tub (Ismail, 1997) while another one involves releasing the earthworms in lukewarm water and agitating them gently so as to shock them to secrete higher amount of body fluids and mucus (Kale, 1998).

Plant-beneficial microorganisms in vermicompost and vermiwash: less researched area

It is well known and widely reported that earthworm casts or vermicompost possess high contents of nitrogen (Bansal and Kapoor, 2000), phosphorus (Parmanik *et al.,* 2010), plant growth hormones such as auxins, cytokinins and vitamin B12 (Tomati *et al., 1983*) and humic acids (Atiyeh *et al.,* 2002; Arancon *et al.,* 2006). Similarly, vermiwash too has been reported to be rich in nutrients and plant growth hormones (Ismail, 1997, Suthar, 2010; Gopal *et al.,* 2010). Their application in improving the yield of horticultural (Gajalakshmi and Abbasi, 2002; Gutiérrez-Miceli *et al.,* 2007; Singh *et al.,* 2008, Gopal *et al.,* 2010, Chaterjee *et al.,* 2012) and agronomic crops (Rajkhowa *et al.,* 2000; Jeyabal and Kuppuswamy, 2001; Tejada and Gonzalez, 2009; Roy *et al.,* 2010, Hatti *et al.,* 2012) are well documented.

However, what is not well studied and remains largely unrecognized is the fact that the vermicompost and vermiwash act as foci for dissemination of microorganisms, particularly the plant-beneficial communities. Few articles report upon the microbial diversity in the finished compost and vermicompost (Fracchia *et al.,* 2006) or the microbial community size, structure and function (Vivas *et al.,* 2009) or isolation of plant-beneficial microbes from compost and vermicompost (Hameeda *et al.,* 2006) to be used as bioinoculant, but none of them characterize the vermicomposts exhaustively for the plant-beneficial microbial community profile.

Plant-beneficial microbes in coconut leaf vermicompost and vermiwash

Coconut (*Cocos nucifera* L.) is an important plantation crop grown in an area of 1.8 million ha in India. The leaf biomass residue produced by this palm is to the tune of 6-8 tonnes annually from one hectare area. High lignin content in the coconut leaves and petioles makes it one of the most recalcitrant organic matters resisting natural decomposition. However, using a local strain of earthworm, *Eudrilus* sp., degradation of the coconut leaves could be hastened to 3 months time and recycled to valuable vermicompost (Prabhu *et al.,* 1998).

An elaborate study was conducted to understand the dynamics of general and plant-beneficial microbial communities during the conversion of coconut leaves mixed with cow-dung to vermicompost using the indigenous earthworm, *Eudrilus* sp. This was compared with the vermicompost produced from cow-dung alone by the same earthworm. Fifteen microbial communities were analyzed in the two sets of substrates (coconut leaf + cow-dung in 10:1 w/w ratio and cow-dung alone), in the gut contents of the *Eudrilus* sp. feeding on the substrates and in the vermicasts excreted by the earthworm. Similarly the vermiwash produced from coconut leaf vermicompost was analyzed for the microbial parameters.

The results of the studies showed that during the vermicomposting process 9 out of 15 microbial communities, particularly the plant beneficial ones were enriched in the vermicompost produced from coconut leaves + cow dung mixture compared to that of 5 communities in vermicompost produced from cow-dung alone. The coconut leaf vermicompost contained significantly high populations of fungi, free-living nitrogen fixers, phosphate solubilizers, fluorescent pseudomonads and silicate solubilizers whereas the cow-dung vermicompost was preponderant with aerobic heterotrophic bacteria; actinomycetes and *Trichoderma* spp. Spore forming and plant growth promoting *Bacillus* spp. were present in similar numbers in both the vermicomposts **(Fig. 1)**. The thickness of the bars in **Fig. 1**., represents log colony forming units g^{-1} dry weight samples analyzed; more the thickness, more the population of that particular microbial community. On the other hand, the bacterial content of coconut leaf vermiwash was low compared to vermicomposts which had 10^{6-8} cfu g^{-1} of sample (Gopal *et al.,* 2009). This may be because of high phenol concentration in the vermiwash, which possesses microbicidal attributes. There is a low probability of the vermiwash getting spoiled since the microbial load is very low. However, within the low counts of plant-beneficial microorganisms in coconut leaf vermiwash, a high population of fluorescent pseudomonads was observed contributing nearly 50% taof the plant-beneficial microbial communities consisting N-fixers, P-solubilizers and *Bacillus* spp. **(Fig. 2)**.

Metagenomic analysis of coconut leaf vermicomposting process using pyrosequencing technology, a next-generation sequencing platform, also revealed the bacterial diversity at higher resolution. Genomic DNA was extracted using Power Soil DNA kit (MoBio, Carlsbad, USA) from the coconut leaf + cow dung substrate at different stages of decomposition and the final mature vermicompost. The bacterial 16^{S} rRNA gene in the genomic DNA was amplified using barcoded primers for pyrosequencing (454 Gs-FLX). The output files from the platform were analyzed using QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso *et al.,* 2010) and MOTHUR (Schloss *et al.,* 2009), two well documented open-source, platform-independent bioinformatics programmes used for assessing the bacterial diversity in any given area, community or ecosystem. The computational analysis generated 68,438 unique sequences and 18,637 OTUs (Operational Taxonomic Units). The16S rRNA amplicons were

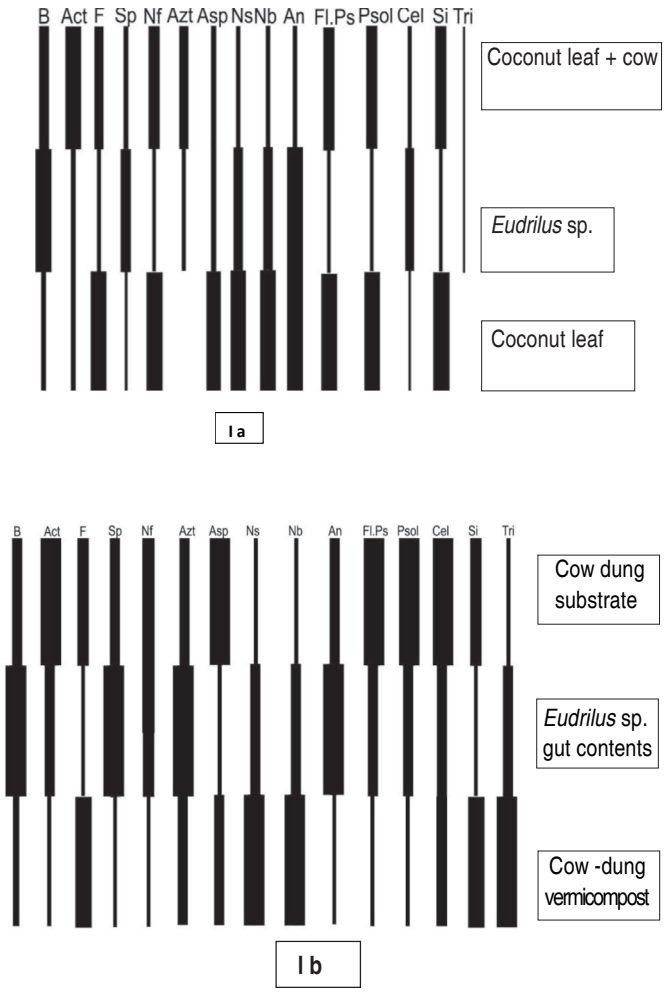


Fig. 1. Qualitative bar coding microbial dynamics during the production of Coconut Leaf Vermicompost (Fig. 1a) and Cow dung Vermicompost (Fig. 1b) from substrate to earthworm gut contents and in vermicompost . (The acronym details- B-bacteria, Act-actinomycetes, F-fungi, Sp-spore formers, Nf-free living N-fixers, Azt-*Azotobacter*, Asp-*Azospirillum*, Ns-*Nitrosomonas*, Nb-*Nitrobacter*, An-Ammonifiers, Fl Ps-fluorescent pseudomonads, Cel-cellulose degraders, Si-silicate solubilizers, Tri-*Trichoderma*)

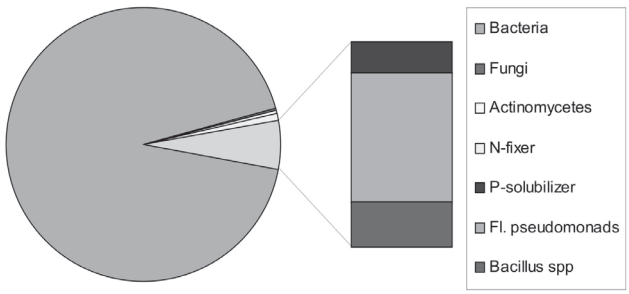


Fig. 2. Pie-chart depicting the microbial composition of coconut leaf vermiwash showing preponderance of plant growth promoting Fluorescent pseudomonads within the plant-beneficial microbial communities

phylogenetically assigned according to their best matches to sequences in SILVA database (Pruesse *et al.*, 2007). The results indicated that the diversity of the bacteria increased as the substrates were converted to vermicompost by the earthworms and then decreased in the finished product to stabilize on par with the initial stage. The α-proteobacteria and Bacteroidetes were the dominant phyla present in the samples followed by γ-proteobacteria and Actinobacteria. Unlike Verrucomicrobia, a significant increase in the Firmicutes numbers was recorded from the first to fourth stage of the vermicompost production (Gopal *et al.*, 2011). These results indicated that the diversity of the bacteria increased as the substrates were converted to vermicompost by the earthworms and then decreased in the finished product to stabilize on par with the initial stage. It has been reported that vermicomposting modifies the original microbial community structure of the substrates in different way and vermicomposting process produces greater bacterial diversity, greater bacterial numbers and greater functional diversity (Vivas *et al*., 2009).

These microbial communities increase the fitness of the soils for sustainable production through (i) effective nutrient mineralization, (ii) inducing systemic and acquired resistance in plants towards pest and diseases, (iii) enhancing the cation exchange capacity of soil and (iv) improving water holding capacity of soil. The results clearly indicated that important groups of microorganisms beneficial for soil health, fertility and plant growth were present abundantly in the coconut leaf vermicompost. Schmidt *et al.* (1997) reported when food was tested as carrier and inoculum source for *Pseudomonas corrugata*, a biocontrol agent, high numbers (10^5-10^7 cfu g^{-1} fresh weight) of the bacteria were recovered from fresh casts of three earthworm species, and similar results were obtained in our earlier work when we inoculated *Metarhizium anisopliae* along with coconut leaves for the control of *Oryctes rhinoceros*, which multiplied in coconut leaf substrate during vermicompost production (Gopal *et al.*, 2006). Both these reports support our observation of increased densities of fluorescent pseudomonads and other beneficial microorganisms during the production of vermicompost from the coconut leaf substrate. Evidences are now coming in confirming our findings that the plant-beneficial microbial communities present in the vermicompost play a key role in imparting systemic resistance to plants against insect pests and diseases (Gopalakrishnan *et al.*, 2011).

Our studies thus highlights the fact that vermicompost not only adds nutrients to the soil, but also large and diverse pool of plant-beneficial microorganisms.

Effect of coconut leaf vermicompost/vermiwash on soil microflora

A soil-incubation study was conducted to evaluate the effect of addition of coconut leaf vermicompost on the microflora of sandy loam soil taken from coconut garden. The following treatments were set up in triplicates (i) soil alone, (ii) coconut leaf vermicompost alone and (iii) soil + coconut leaf vermicompost at recommended dosage of 20 kg/palm. The duration of the study was for 60 days. Sub sampling was done on 0, 15, 30, 45 and 60th day and the observations recorded were:

a. Enumeration of general and plant-beneficial microbial communities in different treatments

b. Estimation of moisture content of the samples and evaluate its effect on the microbial populations

Decimal dilutions of three sample aliquots were inoculated into King's B medium for fluorescent pseudomonads enumeration, Rose Bengal for fungus enumeration, Nutrient agar media for bacteria and bacillus enumeration, Ken Knight and Munaier's medium for actinomycetes enumeration, Pikovskaya's media for phosphate solubilising bacteria enumeration and Jenson's media for nitrogen fixing bacteria enumeration. The inoculated plates were incubated at 36 ^{o}c for 48 hours for bacillus, fluorescent pseudomonads and bacteria, two days for fungi, three days for phosphate solubilising bacteria and six days for actinomycetes and nitrogen fixing bacteria. After incubation, the colonies were counted.

The results of the analysis are given in **Tables 1 & 2**. It is clearly seen that among the three treatments, the coconut leaf vermicompost had the highest microbial load as well as moisture content. Mixing the vermicompost into sandy loam soils significantly increased the population of the microorganisms, particularly, the plant-beneficial ones compared to the soil alone. Moreover, it was seen that, the moisture content of the soil was also maintained at higher level when the vermicompost was mixed into it. The enhanced moisture content helped the microorganisms to mine its nutrient requirement from the substrate and multiply vigorously.

Table 2. Percentage moisture content of samples

	% Moisture content of samples at different incubation period				
Treatments	0 day	15 day	30 day	45 day	60 day
Soil	9	8.3	8.3	8.3	8.0
VC*	57.6	57.6	56.3	56.0	54.6
Soil + VC*	9	9	9	9	8.6

It is clear from the above results that addition of the coconut leaf vermicompost rich in plant beneficial microorganisms increases the same in the soils too. However, to know if really vermicompost added its native plant-beneficial microbial load to that of the soil or if the soil's native plant-beneficial microbial load increased because of addition of vermicompost would require a detailed experiment.

But a molecular analysis with PCR based T-RFLP studies, done usually to understand microbial community structure, conducted in vermicomposts produced from different palm wastes and upon their incubation for 100 days period mixed with sandy loam soil showed that there was a significantly different microbial community structures in the soil and vermicomposts **(Fig. 3)** (Gopal, 2008). The incubated samples showed that microbial community structure moved closer to that of the vermicompost upon mixing with the soil indicating that the addition of vermicompost altered the microbial structure of the soil and pulled it closer to its own structure. However, by the 100th day of the incubation, it was observed that the microbial community structure in soil + coconut leaf vermicompost mixture slowly returned to that present in soil alone **(Fig. 3)**. What emerged from this molecular study was that vermicompost addition significantly altered the microbial community structure of the soil in the initial period. It points to the fact that there was addition of the microbial communities from the vermicompost to soil which precisely proved that vermicomposts are foci for disseminating microorganisms, more so of the plant-beneficial nature that leads to spurt of activities in soil which could improve the mineralization and nutrient availability to the plants, prevent build up of soil pest/pathogens, allow stronger plant establishments besides several other functions that improve the soil fitness for sustainable crop productivity.

Table 1. Effect of addition of coconut leaf vermicompost on the micro flora of sandy loam soil

Treatment	*Bacteria ($n \times 10^6$)*	*Fungi ($n \times 10^6$)*	*Actino-mycetes ($n \times 10^4$)*	*Phosphate solubilizer ($n \times 10^4$)*	*Nitrogen fixers ($n \times 10$)*	*Fluorescent pseudomonades ($n \times 10$)*	*Bacillus spp. ($n \times 10^4$)*
Soil	6.34^{c}	3.98^{c}	6.06^{c}	3.48^{c}	3.20^{c}	0.54^{c}	6.17^{b}
VC	7.73^{a}	4.67^{a}	7.10^{a}	4.48^{a}	3.66^{a}	1.44^{a}	6.66^{a}
Soil + VC	7.13^{b}	4.25^{b}	6.33^{b}	4.22^{b}	3.33^{b}	0.93^{b}	6.59^{a}

Means followed by the same letter in a column, do not different significantly according to Ducan's multiple range test at P=0.05. (log cfu g^{-1} dry sample)

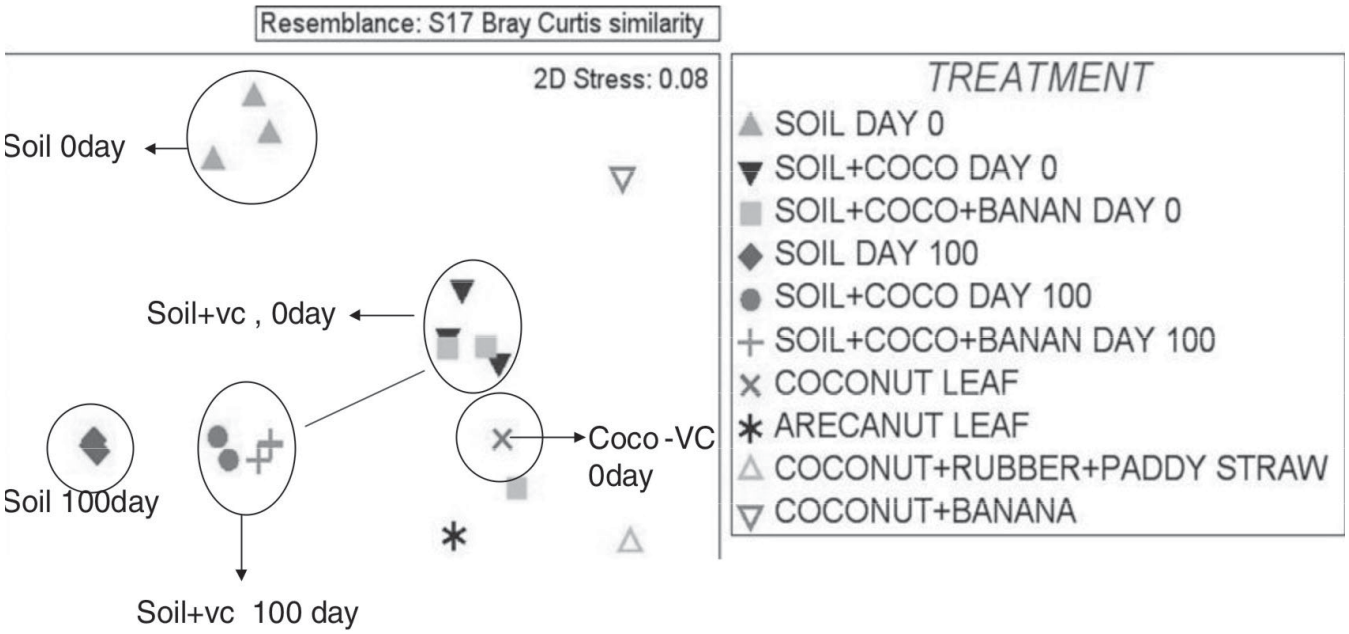


Fig. 3. Changes in the microbial community structure of soil on addition of coconut leaf vermicompost revelaed by T-RFLP studies. The encircled symbols show the interaction effect of addition of coconut leaf vermicompost to sandy loam soils in the incubation studies.

Coconut leaf vermiwash acts in multitude ways to improve soil health and fertility. An important one among them is that it adds the vital fluorescent pseudomonad community to soil besides priming them and other microbial groups to increase in population that carry out critical activities in soil. A field study with application of coconut leaf vermiwash at 1:5, 1:10 and 1:20 dilution in water applied to root region of okra resulted in enhanced microbial populations in the all the treatments compared to the control plot. **(Table 3)**. Several modes of action could be attributed to the enhanced microbial populations such as (a) vermiwash is wash of the composting substrates and of earthworm body rich in the plant growth promoting nutrient content which must be acting as a liquid fertilizer getting immediately and quickly absorbed by the plant roots (b) though the vermiwash by itself was having very low microbial population, on its application in soil, it was observed to potentiate the soil microbial population, particularly the beneficial ones like the nitrogen fixers and the phosphate solubilizers and (c) increase in organic C contents of soil after vermiwash application could boost the rhizodeposition of C from the plants that add to the rhizosphere effect (Gopal *et al.*, 2010).

Coconut leaf vermiwash application also had significant impact on the suppression of the nematode *Meloidogyne incognita* infestation in okra. In the above mentioned field studies it was observed that vermiwash application significantly reduced the gall index, egg mass g^{-1} of root,

Table 3. Effect of coconut leaf vermiwash (CLV) at different dilutions on the rhizosphere microflora (cfu/g dry siol) of okra (*Abelmoschus esculentus)*

	Okra					
Treatment	*Bacteria (n x 10^4)*	*Fungi (n x 10^3)*	*Actino. (n x 10^3)*	*Free N-fixers (n x 10^3)*	*Azospirilla (n x 10^4)*	*Phosphate solubilizers (n x 10^3)*
Pre-treatment	30	14	21	14	5.2	03
Post- treatment						
Control	54	17	30	18	13	02
CLV 1:5	50	29	41	36	160	14
CLV 1:10	91	22	20	23	54	09
CLV 1:20	44	30	18	13	35	03
CD (p=0.05)	15.4	7.7	8.8	6.3	—	4.1

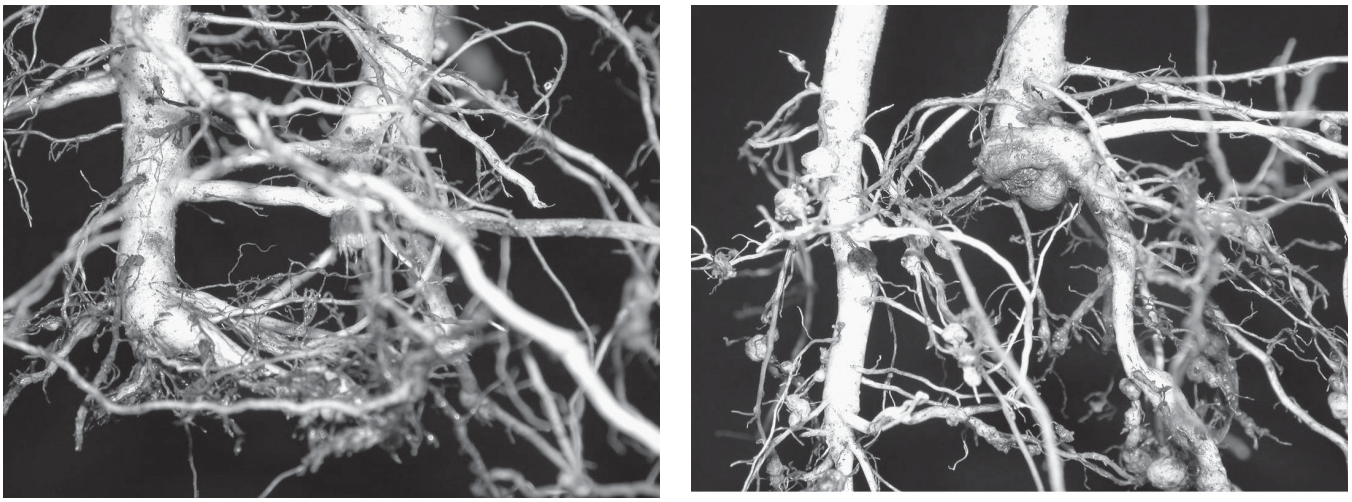
and nematode population g^{-1} of root and per 250 cc soil **(Table 4)**. Upon uprooting the okra for dry weight analysis, the absence or reduction in the number of root galls was clearly seen in the vermiwash applied plants compared to the large number of galls in the control plants **(Fig. 4)**. The coconut leaf vermiwash contains very high counts of fluorescent pseudomonads known for plant growth promotion activities. Though not proved directly, it is possible that these microorganisms present in the vermiwash could have played an important role in the suppression of the nematode population and activities in the rhizosphere of okra. There are several reports that support our presumption that plant growth promoting bacteria, particularly the pseudomonad community, is involved in the suppression of pathogens, pests and nematodes in field conditions (Ramamoorthy *et al.*, 2001; Dong and Zhang, 2006, Kumar *et al.*, 2009). It was also reported that vermiwash contained thiocarbamic acid which was known to possess pesticidal properties (Parmanik, 2010). Possibly, the coconut leaf vermiwash may also contain this pesticide component enabling it to have pest suppression property along with the action of the plant-beneficial communities.

Conclusion

Our studies with coconut leaf vermicompost and vermiwash on their microbiological contents and profiles give ample and convincing proof that these solid and liquid organic fertilizers carry significantly high load of plant-beneficial microorganisms and act as focal point for their dissemination into the soils. Their addition is able to alter the microbial community structure of the soils, increase the microbial populations in the soil, specifically those belonging to plant-beneficial communities, and suppress nematode populace and infestation. The overall effects of their addition result in improving the fitness of the soils and make it sustainable for crop growth.

Table 4. Effect of application of coconut leaf vermiwash on nematode infestation in okra

Treatments	*Gall index*	*Eggs mass /g root*	*Nematode population /g root*	*Nematode population /250 cc soil*
Control	3.0	23	72	230
CLV 1:5	1. 0	2	12	120
CLV 1:10	1.0	11	37	148
CLV 1:20	2.0	17	55	157



4 a. Absence of any nematode caused root galls in vermiwash applied okra plants. **4 b.** Profuse root galls caused by the nematode infestation in control treatment.

Fig. 4. Vermiwash application reduces nematode infestation in okra indicated by absence of root galls (4a) compared to control (4b).

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Research Articles

Optimization of Multiplex RT-PCR assay for detection of potato viruses and viroid with elongation factor 1-α (*ef1α*) as internal control

A. Jeevalatha[1], S. K. Chakrabarti[2], Sanjeev Sharma[1], R. Baswaraj[1] and B.P. Singh[1]

[1]*Central Potato Research Institute, Shimla-171 001, Himachal Pradesh, India*

[2]*Present address: Central Tuber Crops Research Institute, Thiruvananthapuram – 695 017, Kerala, India*

Corresponding author: jeevalatha_a@yahoo.co.in

ABSTRACT

Primer pairs specific to *Potato leafroll virus* (PLRV), *Potato virus X* (PVX), *Potato spindle tuber viroid* (PSTVd) and elongation factor 1-α (*ef1α*) were designed and used in RT-PCR analysis. Based on specificity, amplicon size and annealing temperature one pair of primers for each target were selected for standardization of duplex and multiplex RT-PCR protocols. An expected amplicons of 267 bp, 492 bp, 566 bp and 703 bp sizes specific to PSTVd, PLRV, PVX and *ef1α* of potato, respectively were observed from infected leaves and stored potato tubers both in single and mixed infections. The sensitivity of the protocol was tested with randomly collected field samples, glass house, germplasm collections and tissue culture raised microplants and the protocol was found better than DAS-ELISA. Amplification of the internal control, *ef1α* gene was found throughout the experiments and also in potato tubers stored for more than one month which clearly indicated its use in routine detection of potato viruses in seed production system.

Keywords: Detection, PLRV, PVX, PSTVd, Multiplex RT-PCR, elongation factor 1-α, internal control

INTRODUCTION

Unlike most crops where 'true' seed is used for cultivation, potato is usually propagated through tubers. This vegetative mode of propagation is responsible for gradual build up of viruses thereby reducing the quality of seed which is popularly known as seed degeneration. Out of more than three dozen plant viruses capable of infecting potatoes, only eight are reported to occur in India. They belong to *Potyvirus* (PVY & PVA), *Potexvirus* (PVX), *Polerovirus* (PLRV), *Carlavirus* (PVS & PVM), *Begomovirus* (*Tomato leafcurl New Delhi virus*), and *Tospovirus* (*Groundnut bud necrosis virus*). Besides, one viroid (PSTVd) belonging to *Pospoviroid* and Phytoplasmas (Marginal Flavescence, Purple Top Roll, Hairy Sprout) are also responsible for degeneration. Most of those viruses and virus-like agents are transmitted by insect vectors like aphids, whiteflies, thrips *etc*., that are more active under tropical and subtropical agro climatic conditions of India. The technology of seed production as well as the profile and intensity of viruses and virus like organisms are changing continuously with time.

In vitro techniques are now being favoured for multiplication of potato planting materials. To avoid large scale contamination during micro-propagation, it is necessary that the mother stock should be free from any virus. Efficient and robust techniques for detection of viruses and viroid in potato microplants are therefore, pre-requisites for taking up large scale seed production through tissue culture. ELISA is being used routinely for detection of viruses in seed tubers as well as standing crop. However, lower sensitivity of ELISA techniques necessitates development of PCR protocols for virus detection. RT-PCR analysis is more sensitive but will be more costly when large number of samples are being tested for these viruses separately. Multiplex RT-PCR that employs more than one pair of primers in a single tube for detection of number of viruses simultaneously, can offer a less expensive alternative that will also save analysis time and labour cost. Re-optimization of PCR conditions and reagents is necessary with increasing number of targeted viruses for effective detection and also to reduce non-specific amplification.

At reverse transcription stage, the concentration of various reagents and primer combinations significantly affect the subsequent amplification process, and use of a common antisense primer is reported to overcome this problem (Singh *et al*., 2000). Different strategies like using common degenerate primer (Saade *et al*., 2000), Oligo dT primer (Nie and Singh, 2000; Bostan and Peker, 2009) and

commercially available random hexamer primer (Nie and Singh, 2001; Peiman and Xie, 2006) were tried and found successful. A further modification of multiplex RT-PCR, competitive RT-PCR, with a single-antisense primer and multiple sense primer for the differentiation and detection of different strains of the same virus has been demonstrated (Singh and Nie, 2003). In multiplex RT-PCR, the dNTPs concentration should also be increased at reverse transcription stage for successful amplification from mixed infections (Bariana *et al.*, 1994; Singh *et al*., 2000). At polymerase chain reaction stage, an increased requirement for Mg^{2+}, dNTPs, and *Taq* DNA polymerase and proportion of each primer pair are some of the important factors for successful multiplex PCR.

In case of RT-PCR analysis, false negative results may be produced due to failure in any of the preceding steps like sample extraction, cDNA synthesis, PCR set up. Therefore, use of an internal control will discriminate between a healthy sample and a failed amplification. There are a number of reports on multiplex RT-PCR detection of potato viruses from different parts of plants without or with plant internal control (Nie and Singh, 2000, 2001; Pieman and Xie, 2006; Du *et al*., 2006; Bostan and Peker, 2009). Usually, housekeeping genes are used as internal control since the expression of the genes is influenced by the biotic and abiotic stress conditions. Du *et al*. (2006) compared the sensitivity of 18S ribosomal RNA and *nad2* mRNA as an internal control to simultaneously detect five potato viruses and found that detection of 18S ribosomal RNA is more sensitive than *nad2* mRNA.

Nicot *et al*. (2005) studied the variability of expression of seven housekeeping genes *viz*., actin, aprt, 18S rRNA, *ef1*α, tubulin, cyclophilin and the ribosomal protein L2 for realtime RT-PCR normalization and observed the stable expression of *ef1*α gene in potato both during late blight infection, cold and salt stress conditions. In this study multiplex RT-PCR for the simultaneous detection of the viroid (PSTVd) and two viruses (PLRV, PVX) using *ef1*α gene as internal control. Although a number of reports are available on multiplex RT-PCR detection of potato viruses and to our knowledge this is the first report of use of *ef1*α gene as internal control in multiplex RT-PCR detection of potato viruses.

MATERIALS AND METHODS

Source of plant material

Potato plants infected with PSTVd, PLRV and PVX maintained in glasshouse and infected tubers stored at room temperature in Central Potato Research Institute, Shimla were used for the selection of primers and optimization of multiplex RT-PCR. The samples were confirmed for PLRV and PVX infection through DAS-ELISA by following the methods described by Clark and Adams (1977) and PSTVd through NASH technique (CIP, Peru). The coat protein gene of these viruses and complete RNA sequence of PSTVd were amplified, sequenced using the automated DNA sequencer (ABI PRISM ^{TM} 310 Genetic Analyzer, Applied Biosystems) and confirmed by comparing the sequences in NCBI through BLAST programme. Randomly collected samples from fields, greenhouse and *in vitro* tissue culture plants were used for evaluation of sensitivity of multiplex RT-PCR assay. Since PSTVd is quarantined pathogen in India, samples were also included from germplasm collections as PSTVd positive.

Primer design

Sequences of elongation factor 1-α (*ef1*α) gene from *Solanum tuberosum* (AB061263) were used to design two pairs of primer specific to 621 bp and 703 bp fragment of *ef1*α mRNA. Three pairs of primer specific to PSTVd based on the sequence information of PSTVd isolates maintained in germplasm collections, each four pairs of primer specific to the coat protein region of PLRV and PVX were designed by using the sequence information of Indian isolates of the viruses reported from the institute earlier. The sequences of Indian isolates were aligned with the sequences of isolates reported in NCBI using BioEdit software and conserved regions were selected for designing primers. Some primer pairs were designed by using software Primer 3 Input (version 0.4.0) and some were designed manually and the properties were checked by Oligonucleotide properties calculator software.

Selection of primers

Total RNA was extracted from PSTVd, PLRV and PVX infected leaf samples and tubers using Spectrum TM Plant Total RNA kit (Sigma-Aldrich, Missouri, USA). First strand cDNA was synthesized using Revert aid TM First strand cDNA synthesis kit (Fermentas, Lithuania) using random hexamer primer. Three µl of template RNA, 1 µl of random hexamer primer (100 µM), 4 µl of 5X reaction buffer (250 mM Tris-HCl (pH8.3), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 1 µl of RNase inhibitor (20 u/µl), 2 µl of 10 mM dNTP mix and 1 µl of M-MuLV Reverse transcriptase (200u/ µl) and 8 µl of DEPC treated water was added, mixed gently and incubated at 25 °C for 5 min followed by 60 min at 42 °C and the reaction was terminated by heating at 70 °C for 5 min.

PCR reactions were carried out in 20 µl reaction volume containing 2.0 µl of cDNA, 2.0 µl of 10X PCR buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$), 0.5 µl of 2 mM dNTPs, 0.5 µl of 10µM of respective forward and reverse primers, 0.5 µl of 1.5 U *Taq* DNA polymerase

(GeNei, Bangalore, India) and 14.0 µl of sterile double distilled water. Gradient PCR was performed with different annealing temperatures (nearer to their calculated annealing temperature) using Veriti 96 well thermal cycler (Applied Biosystems). The temperature profile of the PCR cycle was pre-incubation at 94 °C for 5 min., leading to 35 cycles of melting at 94 °C for 1 min., annealing at 55 °C to 64 °C for 1 min and synthesis at 72 °C for 1 min., followed by an extension of 72 °C for 10 mins. The amplified products were analyzed by electrophoresis in a 1% agarose gel in 1X TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH8.0) at 100 volt for 1 hr. Single pair of primer was selected for each target showing single specific amplification of the target. A common annealing temperature (64 °C) was selected at which all the selected primer pairs amplified the targets.

Uniplex and duplex RT-PCR with internal control

Uniplex RT-PCR was performed as mentioned in selection of primers and duplexing with internal control was performed with slight modifications in the PCR mixture. PCR reactions were carried out in 20 µl reaction volume containing 2.0 µl of cDNA, 2.0 µl of 10X PCR buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$), 1.0 µl of 2 mM dNTPs, 0.3 µl of 10µM of EF-FP and EF-RP2, 0.5 µl of 10 µM of target specific forward and reverse primers, 0.5 µl of 1.5 U *Taq* DNA polymerase (GeNei, Bangalore, India) and 12.9 µl of sterile double distilled water.

Optimization of multiplex RT-PCR

A mixture of the two viruses and the viroid were prepared by mixing the cDNA prepared with random hexamer primer (as mentioned in selection of primers) from single infections. Different concentrations of $MgCl_2$ (2.0 mM, 2.5 mM and 3.0 mM) and primer quantity (0.3 to 0.5 µl) were tried during optimization. The concentration of dNTPs were increased from 0.5 to 2.0 µl. Multiplex RT-PCR was carried out with four primer pairs, PSTVd-NFP/PSTVd-NRP, PLRV-CP-FP1/PLRV-CP-RP1, PVX-CP-FP1/PVX-CP-RP1 and EF1-FP/EF1-RP2 and PCR cycle conditions were same as mentioned in selection of primers.

The final optimized 25 µl PCR reaction mixture contained 0.75 µl of cDNA prepared from each of PSTVd, PLRV and PVX infected samples, 2.5 µl of 10X PCR buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl), 2.5 µl of 25 mM $MgCl_2$, 2.0 µl of 2 mM dNTPs, 0.3 µl of 10 µM of EF-FP and EF-RP2, 0.4 µl of 10 µM of virus and viroid specific forward and reverse primers, 0.5 µl of 1.5 U *Taq* DNA polymerase (GeNei, Bangalore, India) and 11.75 µl of sterile double distilled water.

RESULTS AND DISCUSSION

Commercial propagation of potato is normally done vegetatively using 'seed' tubers. Therefore, degeneration of seed stocks due to viruses is common, as vegetative propagation results in the continuity of several viral pathogens. Due to lack of effective treatment protocols, the main approach for the production of virus-free seeds and planting stocks is rejection of infected plants and selection of healthy plants. Thus, the diagnostics of viral infections is a key stage in obtaining virus-free planting material. Serological techniques have been extensively used for detection of potato pathogens. However, sensitivity of most of the serological tests is far below the expected level and as a consequence lower level of virus inoculums often remains undetected in the tubers. RT-PCR protocols have been standardized to detect single virus at a time but this will be expensive, time and labour consuming when large number of samples are tested for all the viruses separately.

Multiplex RT-PCR can overcome this limitation which has a significant advantage of simultaneous detection of several viruses in a single reaction tube. So an attempt was made to develop multiplex RT-PCR protocol for simultaneous detection of PSTVd, PLRV and PVX along with *ef1*α gene of potato. Selection of primer is crucial because it may interfere with amplification of other target and sometimes gives non-specific bands also when a number of primers are added into a single reaction tube. We designed several primer pairs from the conserved regions and selected single primer pair for each target with different amplicon size. Two pairs of primer specific to *ef1*α gene of potato, three pairs of primer specific to PSTVd, each four pairs of primer specific to the coat protein region of PLRV and PVX were designed and used in RT-PCR analysis at their calculated annealing temperature (55 °C to 64 °C). Based on specificity, size and annealing temperature, one pair of primer for each target *viz*., PSTVd-NFP/PSTVd-NRP, PLRV-CP-FP1/PLRV-CP-RP1, PVX-CP-FP1/PVX-CP-RP1 and EF1-FP/EF1-RP2 was selected **(Table 1)** and these primers showed amplification of single specific band (267bp, 492bp, 566bp amplicons) at 64 °C annealing temperature both from infected leaf and stored tuber **(Fig. 1)**. These primers did not show non specific amplification in an optimized PCR reaction mixture.

To avoid false negative results due to poor quality of nucleic acid (presence of PCR inhibitors) and any failure in the reaction mix or PCR cycle, inclusion of internal control is necessary. Developed duplex RT-PCR assay along with *ef1-*α gene as internal control also have been developed. When these primers were used in duplex detection a fine tuning of the reaction mixture was necessary to avoid faint non- specific bands. An expected size of 703 bp fragment

Table 1. Primers selected for optimization of multiplex RT-PCR

S. No.	*Primer*	*Primer sequence*	*Target*	*Amplicon size*
1.	PSTVd- NFP	5'-TCGGAGGAGCGCTTCAGGGATCC-3'	*Potato spindle tuber viroid*	267 bp
2.	PSTVd- NRP	5'-CTGCGGTTCCAAGGGCTAAACACCC-3'		
3.	PLRV-CP-FP1	5'-CTAACAGAGTTCAGCCAGTGGTTA-3'	*Potato leaf roll virus*	492 bp
4.	PLRV-CP-RP1	5'-CGGTATCTGAAGATTTTCCATTTC-3'		
5.	PVX-CP-FP1	5'-TCAACTACCTCAACTACCACGAAA-3'	*Potato virus X*	566 bp
6.	PVX-CP-RP1	5'-GTAATCTTCACAAAGGCAGCAGTT-3'		
7.	EF1-FP	5'-ATTGATGCCCCCGGACACAGAGAC-3'	elongation factor 1-α (*ef1*α)	703 bp
8.	EF1-RP2	5'-CCTTGGCTGGGTCATCCTTGGAG-3'		

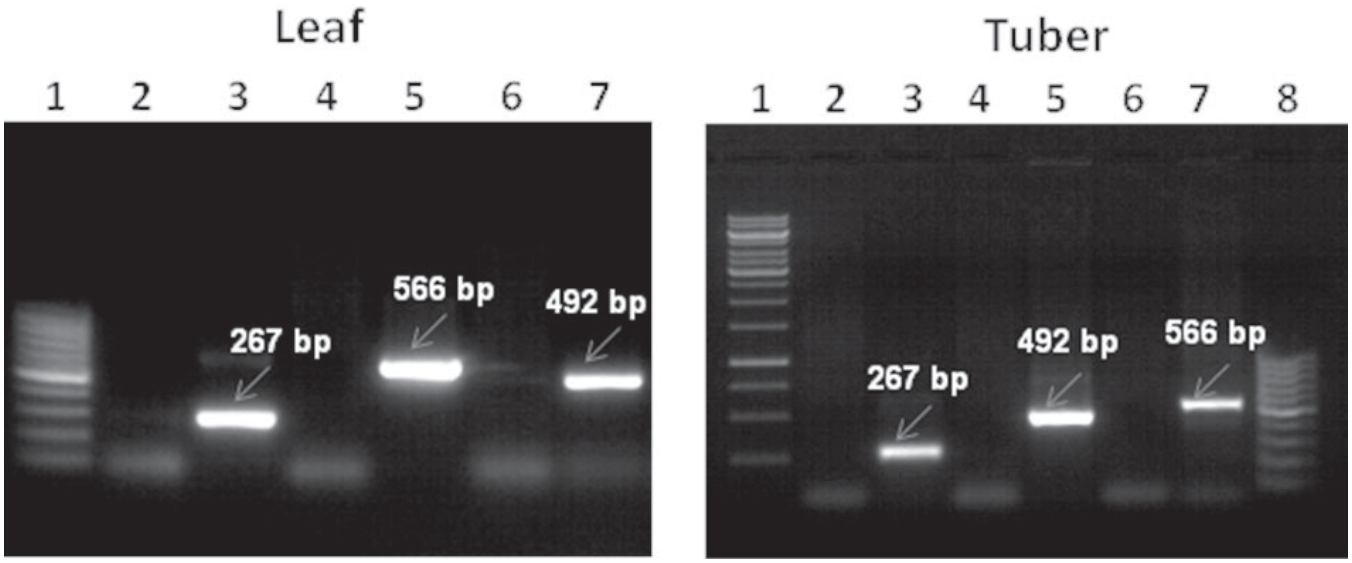

Fig. 1. Uniplex RT-PCR analysis with the selected primer pairs
Lane 1. 100 bp ladder (leaf); Lane 1. 1 kb ladder (tuber); Lane 2, 3. Healthy sample, Infected sample (PSTVd; both in leaf and tuber); Lane 4, 5. Healthy sample, Infected sample (PVX in leaf; PLRV in tuber); Lane 6, 7. Healthy sample, Infected sample (PLRV in leaf; PVX in tuber); Lane 8. 100 bp ladder (tuber)

of *ef1-*α gene was observed in both healthy and infected samples **(Fig. 2)** which indicated the quality of total RNA, successful synthesis of cDNA and PCR reaction. The use of a lesser concentration of *ef1*α primers (final concentration of 150 nM) than virus and viroid specific primers (final concentration of 250 nM) was found optimum to avoid interference of internal control primers in amplification of other targets.

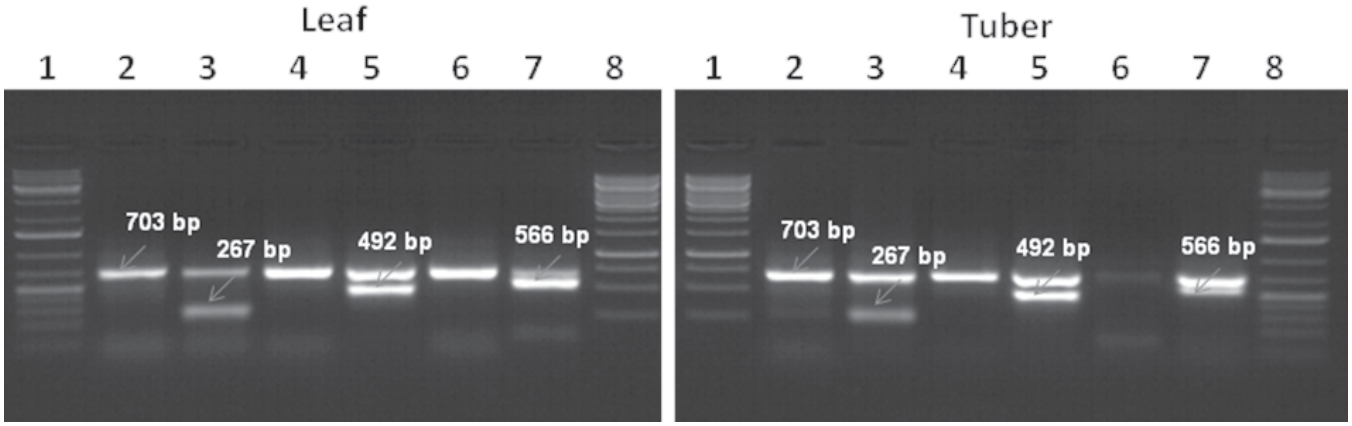

Fig. 2. Duplex RT-PCR detection of PSTVd, PLRV and PVX from leaf and tuber along with *ef1*α gene Lane 1, 8. 1 kb ladder; Lane 2,3. Healthy sample, Infected sample (PSTVd); Lane 4,5. Healthy sample, Infected sample (PLRV); Lane 6,7. Healthy sample, Infected sample (PVX).

Multiplex RT-PCR assay was also standardized for simultaneous detection of PLRV, PVX and PSTVd along with this internal control. During optimization different concentrations of primers, dNTPs and $MgCl_2$ were tested. In PCR reaction mix, 2.0 µl of 2mM dNTPs (final concentration of 160 µM) was found enough to amplify four targets in a single tube. Out of three different concentration of $MgCl_2$ (2.0 mM, 2.5 mM and 3.0 mM), 2.5 mM concentration was found better with clear expected amplicons. The selected primer pairs amplified expected amplicons of 267 bp, 492 bp, 566 bp and 703 bp sizes specific to PSTVd, PLRV, PVX and *ef1*α of potato, respectively from both infected leaves and tubers **(Fig. 3 & 4)** in optimized reaction mix. The optimized protocol was used to test randomly collected from field, glass house,

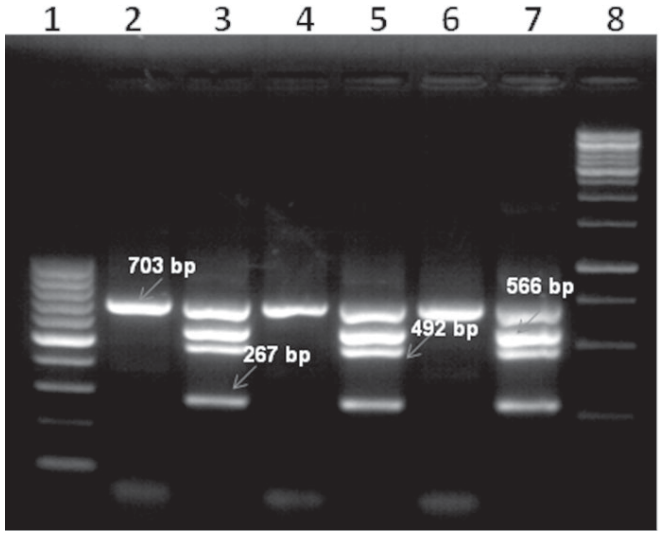

Fig. 3. Multiplex RT-PCR detection of PSTVd, PVX, and PLRV from infected leaves along with *ef1*α gene
Lane 1. 100 bp ladder; Lane 8. 1 Kb ladder; Lane 2, 3. Healthy leaf, Infected leaf (2.0 mM $MgCl_2$); Lane 4,5. Healthy leaf, Infected leaf (2.5 mM $MgCl_2$); Lane 6,7. Healthy leaf, Infected leaf (3.0 mM $MgCl_2$)

germplasm collections and tissue culture raised plants and the results were compared with DAS-ELISA. The sensitivity of the RT-PCR was found greater than DAS-ELISA. RT-PCR could detect PVX and PLRV in micro plants and field samples where DAS-ELISA failed to detect them. These primers are now routinely used in our institute for testing of micro plants, germplasms and other field samples.

Multiplex RT-PCR assay has been successfully used in virus diagnosis by using either common degenerate primer (Saade *et al*., 2000) or Oligo dT primer (Nie and Singh, 2000, Bostan and Peker, 2009) to detect polyadenylated viruses, viruses belonging to same group and to differentiate the strains of the same virus or random hexamer primer (Nie

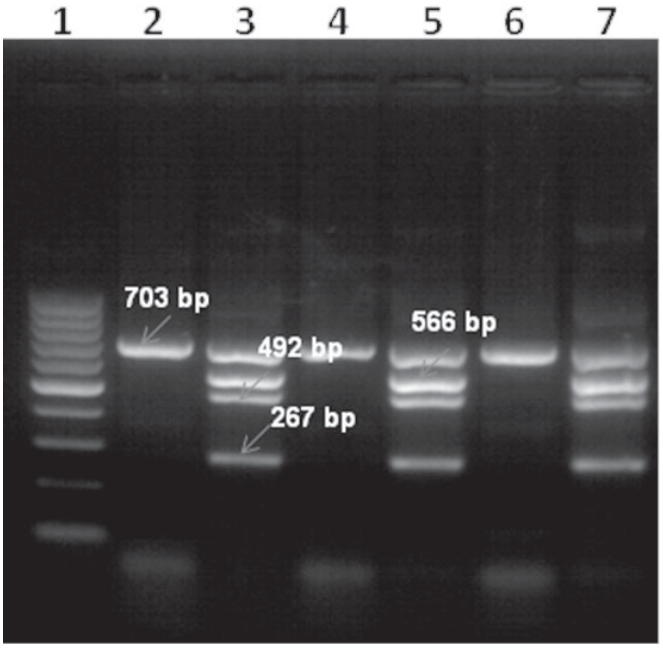

Fig. 4. Multiplex RT-PCR detection of PSTVd, PVX and PLRV from infected tuber along with *ef1*α gene
Lane 1. 1 Kb ladder; Lane 2, 3. Healthy tuber, Infected tuber (2.0 mM $MgCl_2$); Lane 4,5. Healthy tuber, Infected tuber (2.5 mM $MgCl_2$); Lane 6, 7. Healthy tuber, Infected tuber (3.0 mM $MgCl_2$)

and Singh, 2001, Peiman and Xie, 2006) to detect viruses belonging to different groups along with PSTVd. Multiplex RT-PCR and Immuno capture multiplex RT-PCR techniques have been used to differentiate major PVY strains and strain mixtures in a single reaction tube (Lorenzen *et al*., 2006; Ali *et al*., 2010). Matsushita *et al*., (2010) could distinguish between PSTVd and TCDVd in single reaction tube using common reverse primer for first strand cDNA synthesis. Recently, Khan *et al*., (2009) developed multiplex RT-PCR protocol to detect PSTVd in the presence of five potato viruses using random hexamer primer. Since PLRV and PSTVd genomes are not polyadenylated, we have also used random hexamer primers for cDNA synthesis to detect PSTVd with other two potato viruses and internal control.

In this study *ef1*α gene of potato was used as internal control. Nicot *et al*., (2005) used this gene for normalization of potato RNA for realtime PCR analysis. This gene was found as a stable housekeeping gene during both biotic and abiotic stress conditions out of seven housekeeping genes tested in potato by Nicot *et al*., (2005). Expression of this *ef1*α gene during all the experiments in both healthy and infected samples were also found. It shows the reliability of using this gene in detection of potato viruses as internal control. Du *et al*., (2006) compared the sensitivity of 18S ribosomal RNA and *nad2* mRNA as an internal control to simultaneously detect five potato viruses and found that

detection of 18S ribosomal RNA is more sensitive than *nad2* mRNA. The amplification of 18S rRNA was found in samples incubated at room temperature upto 10 days whereas *nad2* was not detectable from samples incubated for more than 8 days. In the study, the amplification of *ef1α* was observed in potato tubers stored for more than one month which indicates that it is better than 18S rRNA for RT-PCR analysis of stored samples. However, the amplification of *ef1α* from fresh leaf samples showed high band intensity compared to stored tubers **(Fig. 3 & Fig. 4).** Similarly, He *et al*., (2006) used Cytochrome oxidase mRNA as an internal control to detect PVY and PLRV from single aphids. Some other genes like mitochondrial *nad5* in apple (Menzel *et al*., 2002), NADH dehydrogenase ND2 subunit (*ndhB* gene) in lettuce crops (Navarro *et al*., 2004), ribulose 1,5-bisphosphate carboxylase chloroplast gene (*Rbcl* gene) in stone fruits (Sanchez- Navarro *et al*., 2005) have also been reported as internal control.

Although the standard multiplex RT-PCR protocols are available for combined detection of potato viruses and PSTVd, here we report multiplex RT-PCR protocol using *ef1α* gene as internal control for the detection of Indian isolates of PLRV and PVX along with PSTVd. This multiplex RT-PCR assay will be useful in selecting virus and viroid free mother plants in high tech seed production systems and parent material for breeding purpose.

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Fertilizer management of cauliflower (*Brassica oleracea var. botrytis*) as influenced by method of raising seedlings, depth of placement and doses of P using ^{32}P-labelled superphosphate

S. C. KOTUR

Indian Institute Horticultural Research, Bangalore, Karnataka –560 089
Corresponding author: sckotur@gmail.com

ABSTRACT

To standardize the fertilizer management of 'Tetris' cauliflower grown by transplanting seedlings nursed in raised bed on soil and in pro-trays using coco-peat medium, compiled with two bandings depths (5 and 10 cm) and three doses of P fertilizer (60, 80 and 100% of recommended dose) were evaluated. Twenty five days-old seedlings nursed on raised bed were distinctly more vigorous in terms of length, girth and dry weight compared to those grown in pro-trays. As a result, the former produced significantly higher yield, dry matter, P content, P uptake as well as fertilizer P utilization. Between the depths of placement of fertilizer, the deeper banding at 10 cm performed better in terms of different parameters of P use by the crop. Increasing P dose increased yield, dry matter and other parameters of P absorption from applied superphosphate. Interaction effect however, showed that both 80 and 100% of recommended P dose applied at 10 cm depth were *at par* and produced significantly higher curd yield when cauliflower seedlings were grown on raised bed. Saving of P input to the extent of 20% was possible when seedlings were grown on raised bed and banding of fertilizer was done at 10 cm depth. The results warranted that the vigour of the seedlings at transplantation stage is crucial for good performance of 'Tetris' cauliflower. Growing seedlings of cauliflower on raised beds using field soil still holds promise till the vigour of the pro-tray seedlings involving fermented coco peat is enhanced *at par* with the former seedlings by appropriate modification.

Keywords: Method of raising seedling, depth of fertilizer application, raised bed seedlings, P-use efficiency, ^{32}P-labelled superphosphate, pro-tray seedlings.

INTRODUCTION

Raising vegetable seedlings in pro-trays using fermented coco-peat has become extremely popular among vegetable growers across the country (Prabhakar *et al.*, 2004). The use of microbial consortium, containing N-fixers, P-solubilizers and growth promoting microbes, to quicken and improve the health of the seedlings in the nursery and in the field after transplantation, has further boosted this activity (Kennedy *et al*., 2004; Anonymous, 2011). Pro-tray grown seedlings produce uniform growth and overcome the initial transplantation shock, are an added advantage especially in hybrid vegetables like tomato and cauliflower with high cost of seed. Since, there is a distinct difference in the seedling vigour between pro-tray seedlings using coco-peat and raised bed seedlings grown in the field (Kotur, 2008), there is a need to refine production practices of crops in general and regarding fertilizer management in particular when grown from pro-tray seedlings. Studies were, therefore, taken up in 'Tetris' cauliflower to evaluate the two kinds of seedlings when ^{32}P-labelled superphosphate was banded at different depths and doses in a typical red loamy soil.

MATERIALS AND METHODS

A field experiment was carried out during December 2009 – February 2010 on a red sandy loam (Typic Haplustalf) belonging to Thyamagondlu series. The soil had a pH of 6.6, organic matter 0.65%, cation exchange capacity 9.2 cmol (p) kg^{-1} and available (Bray-I) P of 2.5 mg kg^{-1}. The experiment was laid out in a completely randomized factorial experiment consisting of 3 factors: (i) 2 methods of growing seedlings on raised bed using field soil and pro-tray using 'Arka fermented'coco peat as the substrate; (ii) banding fertilizers at 2 depths at 5 cm and 10 cm deep furrows and mulching with soil and (iii) 3 levels of P (60, 80 and 100% recommended dose) replicated 4 times. The moulded tray of recycled high impact polystyrene (HIPS) of 27 × 53 cm outer size, had 98 plugs/ holes/ conical cavities of 3.0 × 2.0 × 4.5 cm dimension (volume 20 cm^3), arranged in a grid of 14 × 7 holes with a perforation

provided at the bottom for drainage. Microbial consortium containing N-fixers (*Azotobacter tropicalis*), P-solubilizers (*Bacillus aryabhattai*) and plant growth promoters (*Pseudomonas taiwanensis*), was applied to 6-day old seedlings @10g litre^{-1} of water in pro-trays and @ 20g litre^{-1} in raised beds. The properties of the 2 growing media are presented in **Table 1**. For recording dry weight of shoot and root portions of the seedlings, 10 lots of 10 seedlings each were used. The length of root, shoot and their girth at the beginning of shoot portion was recorded from ten randomly chosen seedlings. Girth was measured using Vernier callipers. The shoot and root portions were separated, washed and dried in oven at 70 °C for 48 hours. The plant parts were analyzed for different nutrients **(Table 2)** adopting standard procedures (Chapman and Pratt, 1961). The seedlings were transplanted at 50 × 60 cm spacing (6 plants plot^{-1}), in vats (plots) of 1.0 × 1.8 m dimension of cement asbestos sheets embedded in soil to a depth of 50 cm to prevent sideward movement of fertilizer nutrients in the soil. The basal dose of fertilizers were banded at the required depths and covered with soil prior to transplantation. Superphosphate was labeled with ^{32}P carrier-free isotope to provide a specific activity of 0.176 m Ci g^{-1} of P. The recommended fertilizer dosage was 180:65:100 NPK kg ha^{-1}. Of this dosage, 50% of N and entire dose of P and K was applied at planting and the remainder of N was top-dressed in a ring around the plant at 5 cm depth, 30 days after planting. Curds of cauliflower were harvested in two lots, and were pooled to determine yield, P content and ^{32}P activity. After the last harvest (85 days after transplanting), the shoot was cut at ground level and separated into leaf and stem, washed with tap- and distilled-water prior to drying in an oven at 70 °C for 48 hours. The samples were ground and wet-ashed in 9:3 (nitric: perchloric) di-acid mixture (Chapman and Pratt, 1961). The activity of ^{32}P in the digest was determined by Cherenkov counting in a liquid scintillation analyzer. Total P was estimated using vanadomolybdate yellow colour method (Jackson, 1967), as a measure of fertilizer P utilization. Phosphorus derived from fertilizer (Pdff, %) was calculated as:

$$\frac{\text{Specific activity of } ^{32}\text{P in the plant part}}{\text{Specific activity of } ^{32}\text{P in superphosphate}} \times 100$$

Phosphorus fertilizer recovery efficiency was calculated as:

$$\frac{\text{P fertilizer uptake by the crop}}{\text{P fertilizer received by the crop}} \times 100$$

Table 1. Fertility status of coco peat (pro-tray) and the soil used to raise 'Arka Ananya' tomato seedlings

Property (Unit)	*Soil*	*Coco peat*
pH (1:2.5 soil:water)	6.6	5.5
Electrical conductivity (dSm^{-1})	0.18	0.67
Organic carbon (%)	0.65	12.5
Cation exchange capacity (cmol kg^{-1})	9.2	32.5
Available N (mg kg^{-1})	221	2545
Available (Bray-1) P (mg kg^{-1})	2.5	10.9
Available K (mg kg^{-1})	95	1246
Exchangeable Ca (cmol kg^{-1})	3.6	7.9
Exchangeable Mg (cmol kg^{-1})	1.0	1.8
Available S (kg^{-1} ha)	20	75
DTPA extractable Fe (μg g^{-1})	23	42
DTPA extractable Mn (μg g^{-1})	27	62
DTPA extractable Zn (μg g^{-1})	3.3	6.0
DTPA extractable Cu (μg g^{-1})	1.9	2.1

Table 2. Composition of 'Tetris' cauliflower seedlings raised on raised bed and in pro-trays

Property (unit)	*Shoot*		*Root*	
	Raised bed	*Pro-tray*	*Raised bed*	*Pro-tray*
Length (cm)	12.07 ± 30512	11.95 ± 1.293	9.651 ±1.305	7.55 ± 1.331
Girth of seedling (mm)	2.60 ± 0.323		2.19 ± 0.186	
Dry matter (mg seedling^{-1})	498 ± 37.9	96 ± 10.7	69 ± 8.9	19 ± 3.4
Nitrogen (%)	2.76 ± 0.025	2.33 ± 0.029	3.16 ± 0.042	1.22 ± 0.013
Phosphorus (%)	0.15 ± 0.003	0.06 ± 0.004	0.11 ± 0.005	0.04 ± 0.005
Potassium (%)	4.37 ± 0.158	3.67 ± 0.225	4.00 ± 0.125	3.56 ± 0.213
Sulphur (%)	0.95 ± 0.019	0.55 ± 0.012	0.31 ± 0.009	0.39 ± 0.012
Calcium (%)	2.22 ± 0.125	1.08 ± 0.044	1.69 ± 0.028	2.09 ± 0.084
Magnesium (%)	0.39 ± 0.009	0.29 ± 0.021	0.38 ± 0.018	0.68 ± 0.019
Iron (μg g^{-1})	153 ± 12.2	56 ± 2.1	540 ± 5.6	368 ± 5.2
Manganese (μg g^{-1})	39 ± 0.6	153 ± 2.4	11 ± 0.9	18 ± 0.8
Zinc (μg g^{-1})	17 ± 0.6	22 ± 0.3	23 ± 0.3	51± 0.9
Copper (μg g^{-1})	2.3 ± 0.09	13.0 ± 0.52	3.4 ± 0.09	7.6 ± 0.13

To verify the results of the experiment and to examine the root characteristics in more detail, the 2 kinds of seedlings were similarly raised in February, 2012 and transplanted in 3 × 2.4 m plots (20 plants/plot) in 3 replications arranged randomly, during the month of January and transplanted in February 2012. Phosphorus was banded at 10 cm depth at 80% recommended dose. At harvest, 3 plants from each plot (9 in all) were carefully uprooted, and the root portion was cut at soil surface, later washing the adhering soil. After recording the dimensions, the secondary roots were separated from the tap root using a scalpel and dried in an oven to obtain dry weight.

RESULTS AND DISCUSSION

Response of seedlings type

The growth and vigour of the seedlings grown in the raised bed were distinctly superior. However, between the two media used for growing the seedlings, coco peat contained decidedly higher quantities of all nutrients as well as a higher load of beneficial microbes as well and was more fertile than the soil used for raised bed seedlings **(Table 1)**. The pH of the substrate, however, was lower (5.5) and it was highly carbonic (12.5%) in nature compared to the pH of 6.6 and organic carbon of 0.65% of the soil. The raised bed seedlings showed similar shoot length, longer root length but were thicker in girth. They produced a higher total dry matter of 594 mg seedling^{-1} **(Table 2)**. Therefore, pro-tray seedlings were lankier and amassed far lesser total dry matter of 115 mg seedling^{-1}. The raised bed seedlings were distinctly healthier and vigorous. They showed generally superior nutrient composition in both the shoot and root portions compared to those raised in pro-trays. It was observed that the raised bed seedlings showed a distinct dominance of tap root **(Fig. 1)** while in the pro-tray seedlings the same was weakened. The same trend was evident in the month of February, 2012 transplanted experiment carried out to study the root growth under the two kinds of seedlings **(Table 3)**. In the crop raised to study the different root growth between the transplants grown from raised bed and pro-tray seedlings, the roots of the former continued to grow vigorously so that the early vigour continued its influence in the advanced stages of crop growth. The root diameter length and dry weight was distinctly higher in the transplants from raised seedling compared to the pro-tray seedlings at harvest **(Table 3)**.

Fig. 1. Pro-tray (left) and raised bed (right) seedlings of 'Trtris' cauliflower seedlings at transplanting

Table 3. Characteristic of roots at harvest in 'Tetris' cauliflower grown from raised bed and pro-tray seedlings when 80% P dose was banded at 10 cm depth (mean of 9 values)

Parameter	*Raised bed seedlings*	*Pro-tray seedlings*
Dry weight of tap root (g)	14.847 ± 0.8812	10.813 ± 0.3353
Dry weight of secondary root (g)	13.124 ± 0.6954	8.280 ± 0.4246
Total weight of the root (g)	27.971 ± 0.790	19.093 ± 0.4214
Length of tap root (cm)	8.39 ± 0.321	8.53 ± 0.279
Girth of stem (cm)	2.8 ± 0.16	2.5 ± 0.16

*Standard deviation (δ_n)

The crop plants obtained from the transplants from the raised bed seedlings produced 35.276t ha^{-1} curd yield, 17.6% higher than obtained by transplanting pro-tray seedlings (30.004t ha^{-1}). Cauliflower grown from raised bed seedlings showed significantly higher curd yield and dry matter, P content, total P uptake as well as fertilizer P utilization of curd as well as the whole plant. On the other hand, phosphorus derived from fertilizer (Pdff, %) of the whole plant in cauliflower grown from raised bed seedlings, was significantly lower owing to dilution of absorbed ^{32}P due to higher dry matter production observed in these plants compared to those grown from pro-tray seedlings.

Depth of placement

Placement of the fertilizer at appropriate depth ensures efficient supply of the fertilizer nutrient to the roots. Between 5 and 10 cm deep placement of fertilizer, the deeper placement showed 13% higher yield of cauliflower curds obtained (34.628t ha^{-1}) than when fertilizer was banded at 5 cm depth. Similarly, dry weight of cauliflower curds was significantly superior when superphosphate was placed at 10 cm depth. However, due to dilution of the absorbed P, the P content of curd and the whole plant showed significantly lower values when superphosphate was applied at 10 cm depth. Different parameters of fertilizer use like phosphorus derived from fertilizer (Pdff, %), fertilizer P uptake as well as fertilizer P utilization were significantly higher when fertilizer was placed 10 cm deep

(Table 4) as the fertilizer was close to the zone of dense roots **(Fig. 2**).

Fig. 2. Roots of plants at harvest grown from pro-tray (left) and raised bed seedlings of 'Tetris' cauliflower

Phosphorus doses

Different P doses were devised to evaluate the exact requirement of the crop for optimum growth and yield. Thus, it was observed that the curd yield, dry matter in curd as well as of the whole plant increased as P level increased from 60 to 100% of recommended P dose **(Table 4)**. The same trend was evident in respect of P content, P uptake, phosphorus derived from fertilizer (Pdff, %) and fertilizer P uptake indicating that 100% of the recommended P dose was necessary to meet the actual needs of cauliflower crop. In respect of fertilizer P utilization, the values decreased as the P doses increased as expected. Because, increasing dosage means, a higher denominator and therefore a lower P utilization. However, the fertilizer utilization of 60 and 80% were *at par* in the case of curd.

Interaction effects

The curd yield was significantly higher at 80% P dose with fertilizer placed at 10 cm depth (38.30t ha^{-1}), 16.1% higher than placement at 5 cm depth (32.98t ha^{-1}). However, at 10 cm deep placement, the curd yield obtained at 80 and 100% P doses were *at par* indicating that a minimum 20% saving of P input is possible to obtain high yield of cauliflower curds **(Table 4)**. The yield differences between the two placements were *at par* at both 60 and 100% P doses. In respect of mean P content, the differences between the two placements of superphosphate at 80% P dose were *at par* in cauliflower grown from pro-trays while in rest of the treatments the trend observed in the main effects prevailed. Phosphorus derived from fertilizer (Pdff, %) values in the whole plant did not change significantly in spite of increasing P doses when P was placed at 5 cm depth in cauliflower grown from pro-tray seedlings. Phosphorus derived from fertilizer values did not show a significant change when superphosphate was placed at 5 cm depth in cauliflower crop raised from both raised-bed and pro-tray seedlings.

Table 4. Effect of kind of seedling, depth of banding and dose of phosphorus on fruit yield, dry matter and composition and parameters of P use efficiency of 'Tetris' cauliflower

Treatment	*Curd yield*	*Dry matter (t ha^{-1})*		*P content (%)*		*P uptake (kg ha^{-1})*		*Pdff (%)*		*Fertilizer P uptake (kg ha^{-1})*		*Fertilizer utilization (%)*	
	(t ha^{-1})	*Curd*	*Total*	*Curd*	*Mean**	*Curd*	*Total*	*Curd*	*Mean**	*Curd*	*Total*	*Curd*	*Total*
Method of raising seedlings													
Raised bed	35.726	2.245	9.542	0.461	0.516	11.322	43.238	13.56	12.71	1.544	5.749	4.43	16.37
Pro-tray	30.000	0.091	8.309	0.452	0.482	10.079	37.529	13.07	13.41	1.244	4.933	3.56	14.30
SEm (±)	0.3811	0.0263	0.0697	0.0044	0.0030	0.1567	0.3678	0.296	0.1286	0.0272	0.0844	0.092	0.263
C.D. (*p*=0.05)	1.1122	0.0768	0.2033	NS	0.0087	0.4572	1.0728	NS	0.375	0.0789	0.2467	0.269	0.769
Depth of fertilizer placement (cm)													
5	30.656	2.100	8.815	0.477	0.508	10.017	40.085	12.56	12.38	1.256	5.185	3.65	14.89
10	34.628	2.447	9.037	0.436	0.490	10.669	39.933	14.07	13.73	1.533	5.318	4.34	15.73
SEm (±)	0.3811	0.0263	0.0697	0.0044	0.0030	0.1547	0.0662	0.296	0.129	0.0272	0.0844	0.092	0.263
C.D. (*p*=0.05)	1.1122	0.0768	NS	0.0129	0.0087	0.4572	NS	0.865	0.375	0.0789	0.2467	0.269	0.769
Level of P dose (% of recommended dose)													
60	30.800	2.221	8.764	0.425	0.442	9.400	33.583	12.09	12.05	1.139	4.333	4.31	16.40
80	32.689	2.249	9.002	0.443	0.499	9.939	40.800	13.3	12.97	1.328	5.378	3.77	15.27
100	34.433	2.351	9.007	0.502	0.555	11.772	45.950	14.56	14.16	1.717	6.306	3.90	14.34
SEm (±)	0.4667	0.0322	0.0854	0.0054	0.0073	0.1917	0.4500	0.363	0.158	0.0328	0.1333	0.113	0.323
C.D. (P = 0.5)	1.3622	0.0941	0.2492	0.0158	0.0107	0.5600	0.3139	1.059	0.460	0.1061	0.3022	0.330	0.942

Table 5. Interaction effect of kind of seedling, depth and level of P on curd yield and total fertilizer P utilization by 'Tetris' cauliflower

Level of P	Raised bed seedling		Pro-tray seedling	
(% of recommended dose)	5 cm	10 cm	5 cm	10 cm
		Curd yield (t ha^{-1})		
60	31.367	33.489	26.728	31.622
80	32.983	38.300	27.933	31.528
100	36.911	38.606	27.994	34.216
SEm (±)		0.9333		
C.D. (P = 0.05)		2.7247		
		Mean P content (%)		
60	0.462	0.431	0.420	0.457
80	0.548	0.497	0.478	0.474
100	0.587	0.574	0.556	0.505
SEm (±)		0.0073		
C.D. (P = 0.05)		0.0214		
		Mean Phosphorus derived from fertilizer (Pdff, %)		
60	11.75	12.38	12.11	11.93
80	11.58	14.23	12.60	13.49
100	12.89	13.43	13.38	16.92
SEm (±)		0.315		
C.D. (P = 0.05)		0.919		

Two important points emerged from this study. Firstly, the mechanical resistance in a positive sense and unrestricted rooting volume that the soil in the raised bed offers are characteristic and encouraged better seedling growth. These factors facilitated normal and healthy growth of roots in general and of the tap root in particular. During the entire duration of growth in the field, as finally shown at harvest, the plants grown from the raised bed seedlings supported a distinctly robust root system in the field also in comparison with the cauliflower plants grown from coco peat raised seedlings. This meant, a substantially higher dry weight, girth and length of the root in the former **(Table 5)**. Conversely, the weaker tap root of the pro-tray seedlings resulted in less vigorous seedlings that went on to grow poorly performing plants in the field upon transplantation. Similar situation was observed in the case of tomato and cabbage hybrid cultivars also (unpublished). However, these results are contrary to that reported in 'Indra' capsicum (Kotur 2008). In capsicum, the pro-tray seedlings were distinctly more vigorous and produced significantly higher yield. The essence of the matter, therefore, hinges on the vigour of the seedling at the time of transplantation of the vegetable crop. The near absence of transplantation shock, notwithstanding, the transplants of pro-tray seedlings performed poorer than the raised bed cauliflower seedlings. Partial damage of the root system of the raised bed seedlings when pulled out from the raised bed before transplanting may also have encouraged the better root- and overall growth of the transplants. Amelioration of coco peat may have to be attended urgently to restore the seedling growth. The implications are serious keeping in view that pro-tray technology is adopted widely in raising seedlings of a large number of crops including tobacco, sugarcane, pigeon pea, marigold and others in addition to the transplanted vegetable crops. Since the performance of two kinds of seedlings is different and is carried forward when transplanted in the field, it is necessary to reassess different crops and their varieties so that the best method of raising seedlings is identified and practised for keeping the production high. Substantial reduction cover 17% cauliflower yields grown from pro-tray seedlings can have significant influence on the profitability of tomato production. The farmers may have to re-adopt the traditional raising of tomato seedlings till nursing of equally vigorous pro-tray seedlings is a reality. Secondly, in cauliflower grown from raised bed seedlings, the yield of cauliflower was distinctly superior when the fertilizer was banded at 10 cm depth **(Table 5 & Fig. 2)**. Better uptake and utilization of applied P was observed in the high yielding variety 'Arka Vikas' tomato, a high yielding but slower growing variety relative to hybrid ones, when superphosphate was banded at 5 cm depth. Then, a saving of 40% P input was possible without significant reduction in yield. In hybrid capsicum, cabbage and cauliflower crops, superphosphate had to be banded at a deeper depth of 10 cm to attain high yield as well as optimum fertilizer use efficiency (Kotur *et al.*, 2006). When judicious P application is practiced, the undesirable build up of P in soil leading to induced Zn deficiency are avoided.

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Recycling coconut leaf-agro wastes mixture using *Eudrilus* sp. and growth promotion properties of coconut leaf vermicompost

George V. Thomas[*], C. Palaniswami[@], Murali Gopal and Alka Gupta

[@]Central Plantation Crops Research Institute, Kasaragod – 671 124, Kerala
[*]Sugarcane Breeding Institute, Coimbatore, Tamil Nadu

Corresponding Author-Email: georgevthomas@yahoo.com

ABSTRACT

The ability of the indigenous coconut leaf vermicomposting earthworm, *Eudrilus* sp., to degrade agro-wastes from pineapple, banana, glyricidia and sugarcane was evaluated individually and in 1:3 ratio (w/w) with coconut leaves. In large-scale trials in tanks, *Eudrilus* sp. was able to convert more than 50% of the agro-wastes tested to vermicompost when mixed with coconut leaves. The mixture of banana pseudostem with coconut leaves yielded highest earthworm recovery followed by coconut leaves alone and coconut leaves + glyricidia mixture. There appeared to be a direct relationship with moisture content of the material to the earthworm multiplication and dry matter content to the vermicompost output. The mixture of banana pseudostem with coconut leaves had higher organic carbon, major and micro nutrient contents compared to coconut leaves alone. The earthworm recovery too was highest in the same combination.

Analysis of the bacterial, actinomycetal and fungal content of the coconut leaf vermicompost and vermicast showed that it contained high numbers of *Arthrobacter, Bacillus*, *Pseudomonas*, *Aspergillus, Actinomyces* and *Streptomyces*. When mixed at 10, 20 and 30% in soil, coconut leaf vermicompost was able to improve the growth and robustness of coconut seedlings. Our findings clearly show the coconut leaf vermicomposting earthworm, *Eudrilus* sp., has broad-spectrum agro-wastes degrading capacity. Thus, the wastes produced from intercrops normally grown in coconut garden can also be mixed with coconut leaves to produce good quality vermicompost in the farm itself.

Keywords: *Eudrilus* sp., vermicompost, coconut leaves, coconut seedlings

INTRODUCTION

Vermicomposting helps in converting the non-available form of plant nutrients present in organic matter to available form through the combined actions of the earthworms and microorganisms (Edwards and Fletcher, 1988). The three main classes of organic wastes suitable for vermicomposting are generated from animal, plant and urban settlements. Among plants, biomass wastes/residues from plantation crops such as coconut (Prabhu *et al*., 1998), arecanut and cocoa (Chowdappa *et al*., 2001), oil-palm (Singh *et al.*, 2011), coffee (Adi and Noor, 2009) and olive (Benitez *et al*., 2002) have been utilized as substrates for vermicompost production.

Coconut palm, a tropical perennial tree crop, is grown in about 1.8 million ha in India. One hectare coconut farm is capable of generating 6-8 tonnes of high-lignin containing biomass wastes annually. Central Plantation Crops Research Institute (CPCRI), Kasaragod had developed an effective technology for recycling coconut leaves to vermicompost using an indigenous *Eudrilus* sp. earthworm (Prabhu *et al.*, 1998). The vermicompost production from coconut leaves can be carried out throughout the year; however efficient turnover can be attained during the monsoon periods (Gopal *et al*., 2004). The vermicompost produced from coconut leaves has been reported as good source of organic manure for improving the soil health and fertility of plantation crops (Thomas *et al*., 2007).

Coconut as mono-crop fetches limited economic return; market trends dictating the price and thereby the farmers' earnings. To overcome this problem, it has been suggested to beneficially utilize the large inter-spaces between coconut palms by growing multi-tiered crops such as pineapple, banana, black pepper, nutmeg and tubers that can generate income regularly round the year **(Fig.1)**. CPCRI has developed a number of highly productive remunerative cropping system models including inter cropping systems, mixed cropping systems and the high density multispecies cropping systems. Also, to enhance the fertility of soil in coconut gardens cultivation of cowpea and mimosa in the basin (Thomas and Shantaram, 1984) and glyricidia

(Subramanian *et al.*, 2005) in the alleys has been recommended. The intercrops also generate voluminous biomass residues, particularly from pineapple and banana.

Fig. 1. A coconut based high density multispecies cropping system with pineapple, banana, black pepper, nutmeg and clove as component crops

The present study was undertaken with the objectives to (i) evaluate the efficiency of coconut leaf vermicomposting earthworm, *Eudrilus* sp., to degrade agro-wastes from inter and mixed crops grown in coconut based cropping systems, (ii) to study the effect of mixing coconut wastes with other agro-wastes on conversion efficiency and quality of vermicompost and finally to (iii) assess the growth promotion ability of coconut leaf vermicompost on coconut seedlings.

MATERIALS AND METHODS

Earthworm species

Epigeic earthworm, *Eudrilus* sp. **(Fig. 2)** earlier isolated from decomposing coconut wastes in CPCRI farm and which was found to be very efficient in decomposing lignin-rich coconut leaf biomass, was used for the experiment. Earthworms were multiplied in coconut leaves + cow dung mixture at 1:1 ratio for a period of 50 days in plastic tubs to obtain sufficient number of earthworms needed for the study.

Trials with individual crop residues

For effective recycling of residues, an attempt was made to evaluate the capability of the coconut leaf degrading *Eudrilus* sp. to use wastes of intercrops grown in coconut garden as substrates. Pineapple wastes, glyricidia leaves, banana wastes (pseudo-stem and leaves) generated from the high density multi-species cropping system plot of the Institute and sugarcane bagasse procured from nearby farm

Fig. 2. The CPCRI strain of *Eudrilus* sp. capable of degrading coconut leaves to vermicompost

were allowed to dry in the sun for few days until maximum moisture was removed. One kg of the dried wastes from each crop was mixed with 100 g of cow dung slurry and put into plastic basins separately. A control treatment of 1 kg dried and chopped coconut leaves with 100g cow-dung were also kept. Water was added to keep the moisture content of the substrates at 50% level. To achieve 50% moisture level, 500 ml of water added to all the treatments except for coconut leaves + banana pseudostem basins to which 300-400 ml water was sufficient as banana pseudostem had good moisture content by itself. The addition of water was done once in 4 days in the coconut leaves and sugarcane bagasse treatments whereas it was once in 5-6 days in the other three treatments (pineapple, glyricidia and banana pseudostem). Owing to their high moisture contents, the three treatments required less frequent watering to maintain 50% moisture content than coconut leaves and sugarcane bagasse. Three replications of each treatment were maintained. The substrates were allowed to pre-decompose for 15 days followed by introduction of 5 adult coconut leaf degrading *Eudrilus* sp. earthworms into each basin. For the next 45 days, water was added to maintain the moisture at 40% level. At the end of the study, the amount of substrate converted to vermicompost, number of worms and their biomass were recorded.

Large scale experiment with mixture of crop residues

Once the ability of the earthworm was tested in small-scale experiment it was scaled up and tried in large vermicompost tanks of dimensions 7 x 2.5 x 1 m (l x b x h). In the Trial 1, taken up during June to August, banana pseudo-stem and glyricidia leaves were evaluated, and in Trial 2, during November to February, pineapple leaves and sugarcane

bagasse were evaluated. Each tank was filled with 750 kg of coconut leaves mixed with 250 kg of other crop wastes along with 100 kg of cow dung slurry. For each treatment, three replicates were maintained, *i.e.* three tanks per treatment. The substrates were kept at 50% moisture by regular addition of water and allowed to pre-decompose for 2 to 3 weeks. To obtain this moisture content status, water was added once in 4 days in similar proportion to the total substrate as in the trials with individual crop residues. This was followed by addition of 1000 adult earthworms into the tanks. At the end of the experiment, when the substrates were converted to vermicompost in 65-75 days time, the total number of earthworms present and the proportion of substrate converted to vermicompost were recorded.

Nutrient analysis of the vermicomposts

All the five vermicompost types using different substrates (coconut leaves, sugarcane bagasse, pineapple wastes, glyricidia, and banana pseudostem) produced in the small scale experiment and three types *i.e.*, coconut leaves alone, coconut leaves + banana pseudostem and coconut leaves + glyricidia in the large scale experient were analyzed for the major, secondary and minor nutrients and organic carbon contents, using standard procedures (Jackson, 1973).

Microbial analysis of vermicompost and vermicast produced from coconut leaves

The bacterial, fungal and actinomycetal distribution in the vermicompost and vermicasts produced from coconut leaves by *Eudrilus* sp. was also analyzed by using selective media, Nutrient agar, Rose Bengal agar and Kuster's agar respectively. Morphologically distinct isolates were purified and identified to genus level in the following manner.

Identification of bacteria

Cultural characteristics, Gram staining, motility test, tests for production of indole from tryptophan, acid production from glucose fermentation by methyl red test, nitrate reduction, hydrolysis of lipid, starch and gelatin, production of catalase and urease enzymes.

Identification of actinomycetes

Cultural characteristics, Gram staining, Acid fast staining, biochemical tests for production of indole from tryptophan, urease, catalase, hydrolysis of starch and gelatin, sugar fermentation and cellulose hydrolysis.

Identification of fungi

Colony morphology, morphological features of vegetative and reproductive structures by staining.

Effect of coconut leaf vermicompost on the growth of coconut seedlings

A poly-bag experiment was carried out to evaluate the effect of coconut leaf vermicompost mixed at 10%, 20% and 30% doses with 20 kg soil on the growth parameters and dry weight production of West Coast Tall (WCT) coconut seedlings. A control treatment without addition of vermicompost was also maintained. Each treatment had 20 replications. Just sprouted seedlings of WCT variety were planted in poly-bag containing the above mentioned soil+vermicompost mixture. The plants were watered uniformly once in four days. After 12 months, the plant height, girth, number of leaves and length of petiole were recorded. Five seedlings were randomly picked from each treatment; uprooted and the dry weight of stem and leaves as well as the roots were recorded.

RESULTS AND DISCUSSION

Perusal of data with individual agro-wastes **(Table 1)** showed preference of the *Eudrilus* sp. in degrading the substrates in the order coconut leaves > sugarcane bagasse > pineapple wastes > glyricidia leaves > banana pseudostem. The multiplication of the earthworm was also highest in coconut leaves followed by sugarcane bagasse. However, in pineapple, banana and glyricidia wastes, no earthworm was observed at the end of the experiment. The experiment

Table 1. Ability of coconut leaf vermicomposting earthworm, *Eudrilus* sp., to degrade other agro-wastes (values are average of three replicates)

Treatment	*Initial weight of substrates (g)*	*Initial no. of worms added*	*Final no. of worms*	*Final biomass of worms (g)*	*Compost generated (g)*	*Percent conversion*
Coconut leaves + cow dung (control)	1100	5	57[a]	25[a]	673[a]	61.0
Sugarcane bagasse + cow dung	1100	5	51[ab]	22[a]	315[b]	29.0
Pineapple wastes + cow dung	1100	5	20 [bc]	7[b]	250[b]	23.0
Glyricida leaves + cow dung	1100	5	0[c]	0[b]	230[b]	21.0
Banana pseudostem + cow dung	1100	5	0[c]	0[b]	177[b]	16.0
CD (P=0.05)	—	—	34.0	10.50	181.27	—

Means followed by the same letter are not significantly different at P $\geq$ 0.05 using analysis of variance and mean separation (LSD)

clearly indicated that the *Eudrilus* sp. was capable of digesting different wastes tried, albeit with different degrees of conversion; vermicompost recovery was significantly high from coconut leaves compared to all other substrates. From this study, it appeared that the conversion rates were directly correlated to the lignocellulose and inversely to the moisture contents of the substrates; coconut leaves and sugarcane bagasse having more of the former was most preferred by the *Eudrilus* sp. Our findings support the work reported by Suthar (2007) which mentions that the quality of the food sources play a key role for preference of growth and reproductive performance of the epigeic earthworms that result in waste recycling to compost. Though not exactly similar, Hayawin *et al.* (2010) tested *Eudrilus euginae*'s capacity to degrade oil palm fibre wastes such as empty fruit bunch (EFB), oil palm frond (OPF) and oil palm trunk (OPT) and found that the EFB was the best substrate for the vermicompost production. In more similar kind of study, Gunadi and Edwards (2003) found that among the cow and pig manure as well as fruit and vegetable wastes from markets, the epigeic earthworm *Eisenia fetida* grew and multiplied well in the pig manure compared to other substrates.

Our work, therefore, is the only kind that tried to evaluate the capability of the *Eudrilus* sp. in degrading agro-waste substrates originating from totally different types of plant sources and the results confirmed the broad spectrum consumption capacity of the coconut leaf degrading earthworm. The major nutrient and the organic carbon contents in the vermicompost produced from small scale experiments are given in **Table 2**. It can be clearly seen that the vermicompost produced from coconut leaves had the highest OC content followed by sugarcane bagasse vermicompost. However, the major plant nutrient contents were highest in the vermicompost produced from glyricidia leaves and from banana pseudostem. The potash content appeared less than the expected level in coconut leaves and sugarcane bagasse. The reason was addition of water more frequently to maintain the moisture to 50% during the vermicomposting process. The addition of water would have leached the water soluble potash thus reducing it in the compost.The trial with individual agro-wastes gave an indication about the capability of the *Eudrilus* sp. for degrading other agro wastes. However, as the conversion was below 50% it was decided to mix the wastes at the rate of 25% with 75% coconut leaves and then evaluate if the earthworms can improve their efficacy in degrading the mixed substrates. The vermicomposting period in the large scale trials was same for all the treatments as coconut leaves was the major component used. The other agro wastes formed only 25% of the substrate on w/w basis. No significant difference in the composting time was observed. However, there was marked difference in the compost turnover and earthworm multiplication. The results of mixing agro-wastes such as pineapple waste, banana pseudo stem and leaves, sugarcane bagasse and glyricidia green manure with coconut leaves in 1:3 proportion on vermicompost production and multiplication of *Eudrilus* sp. is given in **Table 3**. Vermicompost recovery to the tune of 60% and above was recorded when agrowastes were mixed with coconut leaves at 1:3 ratio, w/w basis. The maximum compost recovery was in treatment that had coconut leaves alone and the least in coconut leaves + banana pseudostem. The difference in dry matter and moisture content in the substrates would have resulted in this outcome. Coconut leaves having high dry matter content and lesser moisture content resulted in significantly highest recovery. The treatments coconut leaf + pineapple wastes and coconut leaves + sugarcane bagasse also had compost recovery at par with the coconut leaves treatment. The trials also revealed that mixing of banana pseudo stem and coconut leaves supported significantly highest level of *Eudrilus* multiplication when compared to other agro-wastes tested and the treatment of coconut leaves alone. One of the main reasons for high earthworm recovery in the coconut leaves + banana pseudostem treatment was the presence of optimum moisture content for the worm reproduction and multiplication. Transfer of the biomass from substrates to earthworms' body weight could also be partially responsible for lesser vermicompost recovery in

Table 2. Nutritional characteristics of vermicompost produced from different crop residues

Treatment	*N %*	*P %*	*K %*	*OC %*	*C:N ratio*
Coconut leaves + cow dung (control)	1.38^{b}	0.27^{c}	0.28^{b}	25.4^{a}	18.4^{a}
Sugarcane bagasse + cow dung	1.44^{b}	0.48^{b}	0.46^{b}	23.9^{a}	16.6^{b}
Pineapple wastes + cow dung	1.24^{b}	0.51^{b}	2.10^{a}	16.8^{c}	13.6^{c}
Glyricida leaves + cow dung	2.14^{a}	0.79^{a}	2.69^{a}	20.6^{b}	9.7^{d}
Banana pseudostem + cow dung	1.24^{b}	0.72^{a}	2.91^{a}	16.6^{c}	13.4^{c}
CD (P=0.05)	0.22	0.12	0.81	2.7	1.34

Means followed by the same letter are not significantly different at $P \geq 0.05$ using analysis of variance and mean separation (LSD).

Table 3. Effect of mixing other agro-wastes with coconut leaves on vermicompost production and earthworm multiplication in large scale trial (Values are average of three replicates)

Treatment	*Initial substrate weight (kg)*	*Vermicompost recovery (kg)*	*Earthworm Recovery (No.)*
Coconut leaves	1000	725[a]	6000[b]
Coconut leaves + gyricidia leaves	750+250	650[b]	6000[b]
Coconut leaves + banana pseudostem	750+250	600[c]	10000[a]
Coconut leaves + pineapple wastes	750+250	700[a]	4000b[c]
Coconut leaves + sugarcane bagasse	750+250	700[a]	3000[c]
CD (P=0.05)	—	45	2226

Means followed by the same letter are not significantly different at $P \geq 0.05$ using analysis of variance and mean separation (LSD)

the same treatment.This result was totally different from the individual agro-wastes experiment where we failed to get good recovery. Very high moisture in the compost produced from banana pseudostem alone must have driven the earthworms away from the basin. Similar was the results with glyricidia in the small-scale and large scale studies.The nutritional properties of the mature vermicompost produced from large scale experiments are given in **Table 4**. It could be seen that the major and minor nutrient contents, organic carbon and C:N ratio of the three types of vermicomposts vermicompost, more the number of parameters gave significantly higher values. However, a careful perusal showed that at 4 kg dose, most of the values recorded were higher. Thus, for getting quality coconut seedlings, mixing coconut leaf vermicompost @ 20% with soil will be the ideal dose.It can be concluded that the coconut leaf degrading epigeic earthworm, *Eudrilus* sp., is capable of consuming banana pseudostem, pineapple wastes, glyricidia leaves and sugarcane bagasse when admixed with coconut leaves at 1:3 ratio and produce vermicompost with low C:N,

Table 4. Nutrient composition of vermicompost produced from coconut leaves mixed with other agro-wastes

Treatment	*N (%)*	*P (%)*	*K (%)*	*Ca (%)*	*Mg (%)*	*Cu (ppm)*	*Mn (ppm)*	*Zn (ppm)*	*OC (%)*	*C:N ratio*
Coconut leaves	1.34	0.22	0.41	1.48	0.27	13.20	892.0	86.3	17.15	12.5
Coconut leaves + gyricidia leaves	1.23	0.26	0.48	1.24	0.31	16.70	846.5	83	15.84	13.2
Coconut leaves + banana pseudostem	1.51	0.24	0.29	1.52	0.32	15.5	808.5	90.1	19.96	12.3

are comparable to each other. C: N ratio below 20 is one of the best indicator of maturity of composts and it indicated that the nutrient present could be immediately available to the plants. The distribution and population of various microbial genera in the vermicompost and vermicasts produced from coconut leaves by the *Eudrilus* sp. is given in the **Table 5**. Among bacteria, *Arthrobacter* spp. was predominant in vermicasts and the vermicompost produced from coconut leaves, followed by the *Bacillus* and *Pseudomonas* spp. Among fungi, *Aspergillus* spp. was present in highest number in vermicasts whereas *Trichoderma* was recorded highest in vermicompost. In the case of actinomycetes, the vermicasts seemed to have only the *Actinomyces* and *Streptomyces* in it while the vermicompost had three more genera in addition *viz*. *Micromonospora*, *Nocardia* and *Thermoactinomyces*. The results of poly-bag experiment conducted to evaluate the effect of mixing coconut leaf vermicompost with soils as potting mixture is given in **Table 6**. It can be noted that addition of vermicompost significantly improved various parameters recorded barring the height of the seedling and length of the leaves. Higher the dose of application of the

Table 5. Distribution of various microbial genera (CFU g^{-1} sample) in vermicompost and vermicasts from coconut leaves

Genera	*Vermicompost*	*Vermicasts*
Bacteria		
Bacillus	2×10^6	2×10^6
Xanthomonas	4×10^5	1×10^6
Micrococcus	4×10^6	-
Arthrobacter	16×10^6	4×10^6
Pseudomonas	1×10^6	2×10^6
Flavobacterium	-	1×10^6
Alcaligenes	-	2×10^6
Fungi		
Penicillium	3×10^3	1×10^4
Fusarium	1×10^4	-
Trichoderma	3×10^4	1×10^3
Aspergillus	1×10^3	5×10^4
Mucor	1×10^4	1×10^4
Actinomycetes		
Actinomyces	4×10^5	1×10^5
Streptomyces	1×10^5	1×10^5
Micromonospora	2×10^5	-
Nocardia	2×10^5	-
Thermoactinomyces	1×10^5	-

Table 6. Effect of application of vermicompost on growth parameters of coconut seedlings (Values for dry weight analysis are average of 5 replicates while it is 20 replicates for others)

Treatment	*Height of seedling (m)*	*Girth of seedling (cm)*	*No. of leaves*	*Length of leaves (cm)*	*Breadth of leaves (cm)*	*Stem and leaf dry wt. (gm)*	*Root dry wt. (gm)*
Control (20 kg soil)	2.24[a]	15.90	8.2	97.08	27.50	381	46.0
2 kg VC + 18 kg soil	1.98b[c]	18.27	8.11	87.57	33.55	330	58.0
4 kg VC + 16 kg soil	2.05[b]	18.87	8.81	93.15	34.21	357	65.0
6 kg VC + 14 kg soil	1.85[c]	21.61	9.11	86.28	31.14	419	81.0
CD (p=0.05)	0.18	1.97	0.62	8.27	4.82	NS	NS

Means followed by the same letter are not significantly different at $P \geq 0.05$ using analysis of variance and mean separation (LSD)

high organic carbon and high content of major and micro nutrients. These crops, barring sugarcane, are commonly grown in coconut garden as intercrops. The broad-spectrum substrate degrading capacity of the earthworm, proven in our studies, provides the small and marginal coconut farmers a good option to effectively recycle the wastes to valuable manure at farm level from the biomass wastes generated from main coconut crop as well as the inter crops. The coconut leaf vermicompost harbours high population of microorganisms, many of them with plant-beneficial attributes. Mixing coconut leaf vermicompost @ 20% with soil helps in production of robust coconut seedlings. The utilization of multiple wastes for vermicompost production could become one of the ideal bio-resource management technologies for organic farming in coconut based cropping systems (Thomas *et al*., 2004).

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Genetic polymorphism in Indian strains of *Xanthomonas campestris* pv. *mangiferaeindicae*, m*ango b*acterial canker disease pathogen

Rupesh Kumar Mishra[1], B. K. Pandey[1], Ashutosh Pandey[1], Muthukumar M[2]. and R. Kishun[1]

[1]*Molecular Plant Pathology, Division of Crop Protection, Central Institute for Subtropical Horticulture,Rehmankhera, Lucknow – 227107, India*

[2]*Division of Crop Improvement and Biotechnology, Central Institute for Subtropical Horticulture, Lucknow*
Corresponding author: bkpandeylko@gmail.com

ABSTRACT

Bacterial canker (black spot) incited by *Xanthomonas campestris* pv. *mangiferaeindicae* (*Xcmi*) is an endemic problem in the major mango-growing regions, especially in the area where harvesting coincided with rains. The aim of this study was to characterize *Xcmi* by molecular approaches. Genetic variations in strains of *Xcmi* were observed and measured using *hrp* gene RFLP, RAPD and Rep-PCR. Results of present investigation confirm the presence of diversity within *Xcmi* strains. It is interesting to note that the Rep-PCR technique to be extremely reliable, reproducible, rapid and highly discriminatory in the study of diversity in case of *Xcmi*. The reliable identification of the mango bacterial canker pathogen by DNA amplification of *hrp* gene will greatly facilitate disease diagnosis, which leads to its successful management. This can also be used for ecological and epidemiological studies. Further, the use of oligonucleotide primers for the *hrp* gene cluster region provides certainty for identification of strains of *X. campestris* that may not be possible with other methods.

Keywords: *Xanthomonas campestris* pv. *mangiferaeindicae*, RAPD, RFLP, *hrp* gene, MBCD

INTRODUCTION

Mango (*Mangifera indica* L.) suffers from a number of diseases caused by bacteria, fungi and other agents. The bacterial canker (black spot) incited by *Xanthomonas campestris* pv. *mangiferaeindicae* (*Xcmi*) is an important problem in the major mango-growing regions of the world, i.e., Asia, Southern and Eastern Africa, Western Oceania, and the Indian Ocean (CPC, 2005). Since more than two decades the disease became important in India (Kishun, 1995). This pathogen affects all the above ground plant parts and results in substantial loss in fruit yield and quality and bacterium survives mostly in lesions and on aerial parts of mango (Pruvost and Manicom, 1993; Pruvost and Luisetti, 1991) as well as epiphytes (Kishun and Chand, 1994).

Over 124 monocotyledonous and 268 dicotyledonous plant species are hosts of *Xanthomonas* strains (Leyns *et al.*, 1984). Among the *Xanthomonas* species, *X. campestris* comprises at least 125 different pathovars that are distinguished by the diseases they cause (Bradbury, 1984). Traditional methods for the detection and identification of phytopathogenic Xanthomonads rely on isolating the organism of interest in pure culture and performing predetermined biochemical, serological, and pathological tests (Saettler *et al.*, 1989). Sometimes, non-selective or selective enrichments are required to increase the sensitivity of the isolation, which may be complicated by the presence of fast-growing contaminating bacteria associated with plant tissue (Saettler *et al.*, 1989). Nucleic acid-based techniques have also been applied to the detection and identification of phytopathogenic bacteria (Bereswill *et al*., 1992; Manulis *et al.*, 1991; Schaad *et al*., 1989; Seal *et al*., 1992), including some members of the Xanthomonads. The *hrp* gene clusters that determine hypersensitivity and pathogenicity may be appropriate for selection of probes for detection and identification of phytopathogenic bacteria and has been found in several phytopathogenic bacteria (Boucher *et al.*, 1987; Lindgren *et al.*, 1986). The *hrp* genes of phytopathogenic bacteria are also very similar at the protein level to genes that are involved in the secretion of pathogenicity factors by bacterial pathogens of mammals (Fenselau *et al.*, 1992; Gitaitis *et al*., 1987), but not in opportunistic Xanthomonads (Bonas *et al*., 1991; Stall and Minsavage, 1990).

Generally, in development of control strategies against plant diseases, the main problem encountered is the presence of different strains within a given pathogen population and low ability of different modern techniques to detect this variation. Hence, there is a need to identify this variation using proper techniques for devising better control strategies against the disease. The aim of this study was to characterize *X. campestris pv. mangiferaeindicae* by molecular approaches using *hrp* gene restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and repetitive extra-palindromic sequences (Rep-PCR).

MATERIALS AND METHODS

Collection of pathogen strains

Mango bacterial canker disease (*MBCD*) infected samples were collected from mango fruit, stem, leaves and seedlings **(Fig. 1)** and isolations were made on nutrient agar medium. A total of fifteen strains were purified by single colony transfer, proved pathogenic and designated as *Xcmi*. Out of fifteen pathogenic strains, ten representive *Xcmi* 5, 7, 10, 11, 13, 14, 15, 16, 17 and 18 from different mango cultivars/agroclimatic zones **(Table 1)** were selected for molecular characterization.

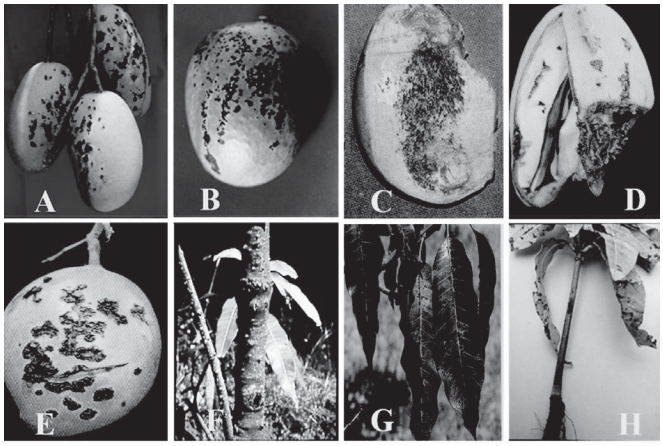

Fig. 1. Disease Symptom's caused by *Xcmi* on mango fruit (A-E), Stem (F), leaves (G) and seedling (H)

DNA extraction

The procedure described by (Kishun and Gupta, 2008) with minor modifications, was used to extract total genomic DNA. Briefly, an aliquot (1.5 ml) of bacterial suspension, grown at 28±1 °C for 48 hrs, pelleted used by centrifugation in a micro-centrifuge (Remi, CPR-12) for 1 min at 14,000 x g. The pellet was washed in 1 ml of distilled water and resuspended in 550 µl TES buffer (50 mM Tris HCl pH 7.8, 50 mM EDTA, 250 mM NaCl). Afterwards 50 µl of SDS (20%, w/v) and 100 µg Proteinase K were added and the mixture was incubated for 1 hr at 55 ºC. Protein and cell debris were extracted with phenol and chloroform mixture (1:24) and the aqueous phase was precipitated with 0.6 volume of isopropanol at -20 °C for 30 min. The pellet was washed in 200 µl of 70% (vol/vol) ethanol and air dried before resuspending in 50 µl of 10 mM Tris HCl (pH 7.8) and 1mM EDTA and stored at -20 °C for further study. DNA concentration was estimated by comparison with known concentrations of Lambda DNA ladder in agarose gel electrophoresis.

Table 1. Collection location (source) of mango bacterial canker pathogen (*Xcmi*) strains of India

S. No.	*Culture I.D.*	*Source*
1.	*Xcmi* 5	CISH, Rehmankhera, Lucknow, Uttar Pradesh
2.	*Xcmi* 7	Projeny orchard, Malihabad, Lucknow, Uttar Pradesh
3.	*Xcmi* 10	IIHR, Bangalore, Karnataka
4.	*Xcmi* 11	CHES, Ranchi, Jharkhand
5.	*Xcmi* 13	Farmer's field, Sabour, Bihar
6.	*Xcmi* 14	Horticulture Garden, Sabour, Bihar
7.	*Xcmi* 15	CHES, Ranchi –I, Jharkhand
8.	*Xcmi* 16	CHES, Ranchi –II, Jharkhand
9.	*Xcmi* 17	BAU, Ranchi, Jharkhand
10.	*Xcmi* 18	RAU Pusa, Bihar

PCR amplification of *hrp* gene

The primer, RST21 (5'-GCACGCTCCAGATCAGCATCGAGG-3') and RST22 (5'-GGCATCGCATGCGCTC TCCGA-3') amplify a 1,075 bp region of *hrp* complementation groups B and C/D of *X. campestris* pv. *vesicatoria*, (Leite *et al.*, 1994*a*) were selected for the amplification of the *hrp* gene region of *Xcmi* strains. Amplification was carried out in 25 µl reaction mixture containing 2.5 µl 1x PCR buffer, 2.5 mM $MgCl_2$, 0.5 mM of each dNTPs, 0.5 µM of each primers, 1.25U *Taq* polymerase (Fermentas) and 10 ng genomic DNA. Amplification was performed with an Eppendorf Thermal Cycler in a programme comprising 34 cycles of denaturation at 94 ºC for 60s, annealing at 61 ºC for 45s, and extension at 72 ºC for 1 min with an initial denaturation of 10 min at 94 ºC before cycling and final extension of 5 min at 72 ºC after cycling. The PCR products were analysed by agarose gel (1%) electrophoresis.

Restriction analysis of *hrp* gene

The DNA fragments amplified from different *Xcmi* strains were restricted with the restriction endonucleases *Alu*I and *Taq*I under conditions specified by the manufacturer (Fermentas). The restricted fragments were separated by agarose gel (2.5%) electrophoresis and gel image was recorded for further studies. DNA restriction fragment

patterns were determined by direct comparison of the electrophoretic patterns of the DNA restricted with each of the four endonucleases. The codes 1 and 0 were assigned according to the presence or absence of each fragment, respectively.

RAPD-PCR fingerprinting

RAPD primers **(Table 2)** were used in Eppendorf Master Cycler. RAPD-PCR was performed in 25 µl reaction volume containing 10 ng genomic DNA, 0.5 µl (10 pmole) primer, 1 µl dNTPs (2.5 mM), 2.5 µl of 10 x buffer with $MgCl_2$ (15 mM), 0.25 µl (5U/µl) of *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd.). DNA was amplified by Eppendorf Master Cycler programme to provide first denaturation for 5 min at 94 ºC followed by 35 cycles of 1 min each at 94 ºC, annealing at 35 ºC for 1 min, followed by 2 min at 72 ºC and final extension for 5 min at same temperature.

Cluster analysis

Comparison of each profile for each primer was carried out on the basis of presence (1) or absence (0) of amplified PCR products of the same length. Bands of same length were scored as identical. A dendrogram was derived from the distance matrix by the Unweighted Pair-Group Method Arithmetic Average (UPGMA) obtained by the program package NTSYS-pc 2.02e (Numerical Taxonomy and Multivariate Analysis System).

RESULTS AND DISCUSSION

PCR amplification of *hrp* gene and RFLP analysis

The 1,075-bp fragments of the *hrp* gene cluster in *Xcmi* strains were amplified with primer RST21 and RST22. The DNA fragments were amplified from all the 10 strains of *Xcmi* with a common size of band pattern of 1,075 bp. The same size fragments were also successfully amplified from DNA of all strains of the other pathovars of *X. campestris* as reported by (Leite *et al.*, 1994*b*). The fragments amplified from strains of *Xcmi* were examined by restriction endonuclease analysis with endonucleases, *Alu*I and *Taq*I. Restriction fragment length polymorphism yielded different restriction patterns with both endonucleases. Although variability was also observed in the banding patterns obtained with the endonucleases *Alu*I and *Taq*I. Differences in the number of common restriction fragments from the amplified DNA of the *hrp* gene cluster indicated the variation in the relatedness of the *hrp* genes of the different strains of *Xcmi*. The restriction fragment data of the *hrp* genes were used to construct dendrogram **(Fig. 2)** based on a parsimony criterion by using UPGMA programme (Rohlf, 1998). Three major groups are generated with 100% similarity within *Xcmi*-5, 10, 16 and 17 and found with closest 33% to the *Xcmi*-18. Strains *Xcmi*-7 and 11 has similar restriction fragment and lies in another group with *Xcmi*-15 having 67% similarities. However, the *Xcmi*-13

Table 2. Sequences of reliable RAPD primers and the number of scorable polymorphic bands of each primer

S. No.	*Primer name*	*Sequence detail*	*No. of amplified bands*	*No. of Polymorphic bands*	*Polymorphic ratio (%)*
1.	OPD-18	GAGAGCCAAC	8	5	62.50
2.	OPD-19	CTGGGGACTT	11	10	90.91
3.	OPE-01	CCCAAGGTCC	7	3	42.85
4.	OPE-08	TCACCACGGT	6	4	66.66
5.	OPF-20	GGTCTAGAGG	9	5	55.56
6.	OPJ-20	AAGCGGCCTC	12	10	83.33
Average	-	-	8.83	6.17	69.81
Total	-	-	53	37	-

Rep-PCR fingerprinting

DNA from the bacteria was subjected to rep-PCR genomic fingerprinting using primer set corresponding to the REP elements of (Versalovic *et al.*, 1991), REP-1R (5'-IIIICGICGICATCIGGC-3') and REP-2I (5'-ICGICTT ATCIGGCCTAC-3'). The rep-PCR protocols were carried out as per methods of (Rademaker *et al.*, 1997). 50 ng of purified DNA was used as template in a 25 µl reaction mixture containing 2 µl (10 pmole) of each primer, 2µl dNTPs (2.5 mM), and 1.25 units of *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd.) in a reaction buffer containing 2.5 µl of 10 x buffer with $MgCl_2$ (15 mM), 30 mM 2-mercaptoethanol and 5% DMSO (v/v). PCR amplifications were performed in a Eppendorf thermo-cycler using the following conditions: an initial denaturation at 95 ºC for 7 min, 35 cycles consisting of 94 ºC for 3s, 92 °C for 30s, then 40 °C for 1 min, extension at 65 °C for 8 min, and a single final extension at 65 °C for 8 min, followed by cooling at 4 °C. PCR products were resolved by horizontal electrophoresis using agarose gel (2%) with TBE buffer (1%) containing ethidium bromide.

and 14 completely separated from all the strains with minimum similarity of 25 per cent.

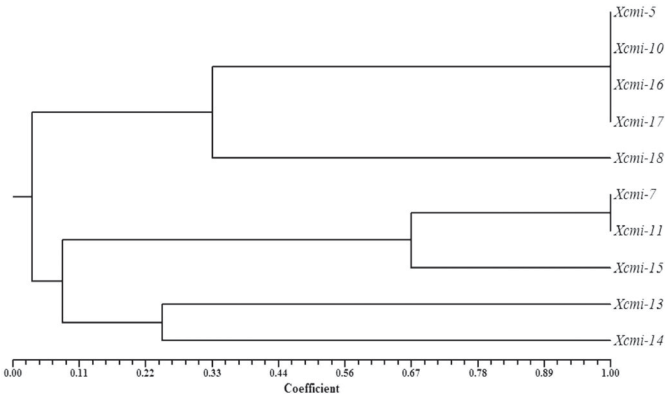

Fig. 2. Cluster diagram based on genetic distance calculated from presence (1) and absence (0) of polymorphic PCR based markers across ten *hrp* gene RFLP of *Xcmi* using the UPGMA

Despite differences in the pathogenic characteristics of these strains (Graham and Gottwald, 1990; Graham *et al*., 1990), the presence of *hrp* gene cluster supports the pathogenic nature of strains. If, they are opportunistic Xanthomonads (Gitaitis *et al*., 1987), there would be lack of *hrp* region (Bonas *et al*., 1991; Stall and Minsavage, 1990). Although the DNA fragments amplified with RST21 and RST22 primer were of the same size for all the strains of *X. campestris*, characteristic restriction banding patterns for each bacterial group occurred with 1,075-bp fragments. Complementation group C/D of the *hrp* gene cluster, from which the fragments were amplified highly conserved among phytopathogenic Xanthomonads (Bonas, 1991). Therefore, the homology of the restriction enzyme fragments from amplified *hrp* genes should furnish valid relationships among these pathogens.

RAPD analysis

Six random primers OPD- 18, OPD- 19, OPE- 01, OPE- 08, OPF- 20 and OPJ- 20 **(Table 2)** were tested with genomic DNA samples of 10 *Xcmi* strains. Out of these six primers screened OPD-19 and OPJ-20 produced amplicons ranging from 280 bp to 1,750 bp with distinct polymorphism **(Fig. 3)**. A total of 53 amplified bands were scored against 10 *Xcmi* strains with RAPD primers. Polymorphism also found in individual strains but this polymorphism was statistically non-significant among the samples. The data revelled by RAPD analysis shows 69.81% variability among these pathogenic strains. Average no. of polymorphic bands was scored and was found to be 6.17 per cent.

The RAPD pattern generated in this study is able to distinguish the differences within the same species, i.e.,

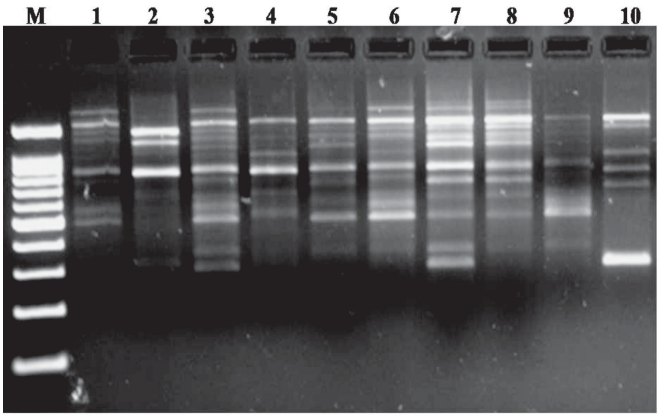

Fig. 3. Representative RAPD profiles of *X. cax*

Xcmi. The quantitative estimate of genetic polymorphism obtained showed a significant level of genetic diversity. Though RAPD has not been used in the evaluation of homogeneity among *Xcmi* strains but carbohydrate utilization profiles (Kishun, 1999) and *hrp*-RFLP study (Pruvost *et al*., 1998) showed almost similar trend. It emanates from present investigation that the molecular tools (PCR-RAPD) could be used for precise and reliable analysis of genetic variability in *Xcmi* strains.

Rep-PCR analysis

The size of the amplification products ranged from approximately 300 bp to 1,000 bp when Rep-PCR fingerprint patterns for *Xcmi* strains were examined. In analyzing the reproducibility of the rep-PCR, comparison of the resulting fingerprint patterns yielded a similarity of approximately 85 to 95% when the patterns were generated in the same PCR experiment and resolved on the same gel. Fragments generated by Rep primer set, were used for the construction of dendrogram. Thus, amplification reactions generated a sufficient number of distinct polymorphic bands for reliable strain discrimination **(Fig. 4)**.

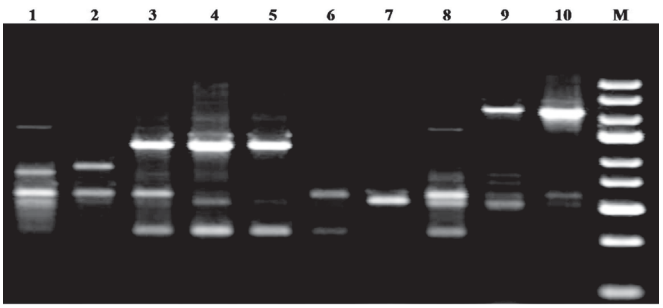

Fig. 4. Agarose gel electrophoresis of PCR fingerprint patterns obtained in 10 *Xcmi* strains using primers corresponding to Rep-PCR. (Lane 1- 10: *Xcmi* strains, Lane M: 100 bp DNA ladder)

The Rep-PCR fingerprints differentiated the *Xcmi* strains which produced unique profiles. Rep-PCR fingerprinting can be considered an important tool for identifying and monitoring the diversity of this pathogen in the affected areas (Trindade Loiselene *et al.*, 2005). In the genus *Xanthomonas*, Rep-PCR has revealed both inter and intrapathovar variability (Louws *et al*., 1994). The studies of Louws *et al.* (1995) with *Xanthomonas campestris* pv. *vesicatoria*, confirmed the separation of this pathovar into two genetically distinct groups, thus confirming the taxonomic studies of (Vauterin *et al.*, 1995). Genetic variations in different isolates of several *Xanthomonas campestris* pathovars were observed and measured using different molecular techniques such as RFLP and Rep-PCR (Bragrad *et al.*, 1995; Louws *et al.*, 1995; Norman *et al.*, 1999; Restrepo *et al.*, 2000).

The *Xcmi* strains showed diversity among themselves with RFLP, RAPD and Rep-PCR fingerprinting analysis. Further, the data of all the three methods were combined to find out more genetic diversity study and a dendrogram generated **(Fig. 5)**. The dendrogram separated the *Xcmi* strains in two major groups consisting similarity of 91% between *Xcmi*-5 and *Xcmi*-7. Genetic similarity coefficient matrix of the *Xcmi* strains was calculated from RFLP patterns of *hrp* gene, RAPD and Rep-PCR and showed combined relationship among the strains **(Table 3)**.

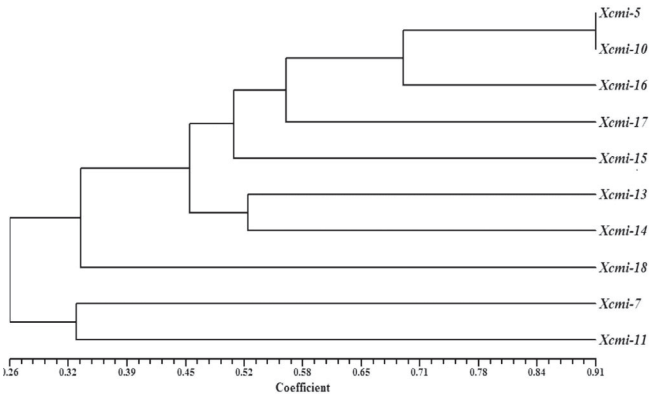

Fig. 5. Dendrogram of 10 *Xcmi* strains derived from the combined data of RFLP, RAPD and Rep-PCR fingerprints by UPGMA based on genetic distance calculated from presence (1) and absence (0) of polymorphic bands

The dendrograms generated from PCR-RFLP of *hrp* gene, RAPD and Rep-PCR analysis clearly indicates the diversity among *Xcmi* strains. Initially, all strains were analysed by RFLP of PCR amplified *hrp* gene of *Xcmi*, isolated from different agroclimatic zones and considerable diversity observed among the RFLP patterns undoubtedly showed that all the strains were very distinct. However, in order to confirm this statement and to clarify the status of these strains, further genetic study is needed. After the RFLP strains were characterised by two other DNA fingerprinting methods *viz*., RAPD and Rep-PCR, the profiles of which enabled strain differentiation and demonstrated a considerable degree of genetic diversity among *Xcmi*. RAPD markers can be a quick and reliable alternative for differentiating the pathogen. RAPD applied to bacterial studies would be useful in providing markers for identification purpose, further revealed the polymorphism within reference strains of *Xcmi* and established DNA fingerprints will be useful for genetic characterization and specific identification of *Xcmi*.

In the present study, dendrograms obtained with individual method showed almost similar pattern of separation, when compared to the combined analysis. Of the three sets of method tested, Rep-PCR was the most conserved, identifying a smaller number of subgroups among the strains. The combined analysis of the patterns generated by RFLP, RAPD and Rep-PCR showed the separation into two groups with 26% similarity **(Fig. 5)**. The molecular weight of the bands in each lane was taken for analysis. The entire two group contained strains collected from different states showed higher degree of region specific genetic variability. The first group composed of only *Xcmi*-

Table 3. Genetic similarity coefficient matrix among 10 strains of *Xanthomonas campestris* pv. *mangiferaeindicae* calculated from RFLP patterns of *hrp* gene, RAPD and Rep-PCR

Strain	*Xcmi-5*	*Xcmi-7*	*Xcmi-10*	*Xcmi-11*	*Xcmi-13*	*Xcmi-14*	*Xcmi-15*	*Xcmi-16*	*Xcmi-17*	*Xcmi-18*
Xcmi-5	1.0000000									
Xcmi-7	0.2307692	1.0000000								
Xcmi-10	0.9090909	0.1851852	1.0000000							
Xcmi-11	0.3076923	0.3333333	0.3076923	1.0000000						
Xcmi-13	0.5000000	0.1818182	0.5652174	0.3333333	1.0000000					
Xcmi-14	0.5200000	0.2727273	0.5200000	0.4285714	0.5238095	1.0000000				
Xcmi-15	0.4615385	0.2727273	0.5200000	0.3636364	0.3913043	0.4782609	1.0000000			
Xcmi-16	0.6956522	0.1600000	0.6956522	0.1923077	0.4347826	0.4583333	0.5909091	1.0000000		
Xcmi-17	0.5600000	0.2083333	0.5000000	0.1481481	0.3200000	0.4000000	0.4583333	0.6363636	1.0000000	
Xcmi-18	0.3600000	0.2631579	0.3076923	0.3000000	0.2173913	0.3636364	0.3636364	0.3478261	0.4090909	1.0000000

7 *and Xcmi*-11 isolated from CISH, Rehmankhera, Lucknow and CHES Ranchi, respectively. The second and largest group included all, *i.e. Xcmi*-5, 10, 11, 13, 14, 15, 16, 17 and 18 strains.

DNA fingerprinting methods used in this study revealed high level of genetic diversity among strains. Results of present investigation confirms the presence of diversity within *Xcmi* strains. It is interesting to note that the Rep-PCR technique to be extremely reliable, reproducible, rapid and highly discriminatory in the study of diversity in case of *Xcmi*. It also confirms the utility of Rep-PCR for differentiation of closely-related strains of bacteria and its potential usefulness to carry out investigation on bacterial evolution in specific ecological areas. The unique fingerprint profiles of *Xcmi* generated by Rep-PCR could be a useful tool in diagnosis and in differentiation of strains. It would be quite useful in the identification of *Xcmi* in tests of routines in plant pathology laboratories. A rapid detection method for pathogens and a diagnostic assay for disease would facilitate early detection of pathogen and lead to more effective management practices.

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Evaluation of INM in citrus (*Nagpur mandarin*): Biometric response, soil carbon and nutrient dynamics

A.K. Srivastava, Shyam Singh, and A.D. Huchche

National Research Centre for Citrus,
Amravati Road, Nagpur - 440 010, Maharashtra, India
Corresponding author: aksrivas2007@gmail.com

ABSTRACT

Emerging multiple nutrient deficiencies have necessitated renewed efforts to address nutrient management issues and integrated use of inorganic fertilizers through organic manures and microbial biofertilizers. Accrued long term field data showed much better effectiveness of microbial consortium (MC) when used in combination with inorganic fertilizers and organic manures, FYM or vermicompost, however latter could produce much higher magnitude of response. The net increase in canopy volume within three years (2007-11) with 100% RDF was 1.54 m^3 compared to 2.34 m^3 with 75% RDF plus 25% vermicompost (Vm) plus MC, with a much favourable fruit quality parameters (48.8% juice, 9.7% TSS and 0.70% acidity). Soil quality parameters in terms of soil microbial biomass (SMB) was much higher (68 x 10^3 / g soil as bacterial count and 41 x 10^3 / g soil as fungal count) and soil microbial biomass nutrient (SMBN) (202.5 mg/kg C_{mic}, 49.4 mg/kg N_{mic} and 24.5 mg/kg P_{mic}) with 75% RDF plus 25% Vm plus MC compared to SMB (32 x 10^3 / g soil as bacterial count and 16 x 10^3 / g soil as fungal count) and SMBN (152.1 mg/kg C_{mic}, 19.1 mg/kg N_{mic} and 16.1 mg/kg P_{mic}) with exclusive use of inorganic fertilizers as 100% RDF. These changes within rhizosphere were very well translated into consequent improvements in leaf nutrient composition, being significantly higher (2.28% N,0.12% P,1.42% K,36.4 ppm Fe – 32.8 ppm Mn – 7.3 ppm Cu – 22.6 ppm Zn) with 75% RDF plus 25% Vm plus MC over 100% RDF (2.13 % N,0.08% P,1.23% K, 30.3 ppm Fe, 28.4 ppm Mn, 7.3 ppm Cu, 19.3 ppm Zn). These observations lend strong support in favour of INM-based treatments than sole use of inorganic fertilizers in the context of offering some restraint of menacing nutrient mining induced decline in citrus orchard productivity.

Keywords: Nagpur mandarin, canopy growth, yield, quality, soil microbial biomass, soil microbial biomass nutrients, soil carbon fractions, leaf nutrient composition

INTRODUCTION

Citrus by the virtue of its extensive cultivation as a key perennial crop in the world trade, has attracted worldwide investigation from various angles. The present citrus production trends are characterized by either frequent crop failure or recurrence of alternate on-and-off years, setting unaccountable monetary loss to the industry (Rojas, 1998). In recent years, nutrient additions have been exclusively in favour of mineral fertilizers due to demographic pressure, demands related to life styles and trade involvement. While the quick and substantial response to fruit yield due to mineral fertilizers eclipsed the use of organic manures, the inadequate supply of the later sources exacerbated this change (Srivastava, 2009). Differential efficacy of two conventional methods of fertilization (soil versus foliar application) has, although helped in improving the quality citrus, but of late, continuous fertilization has failed to sustain the same yield expectancy on a long term basis due to depletion of soil carbon stock and consequently, emerged multiple nutrient deficiencies, irrespective of soil type. The menace of multiple nutrient deficiencies is further triggered through increase in air temperature via changes in microbial communities and activities within the rhizosphere in the light of climate change. Such changes will dictate adversely on the orchard's productive life in long run. Gradual shift from purely inorganic to organic fertilizers started gaining wide scale use for enhanced nutrient cycling (Srivastava *et al.*, 2002).

Integrated Nutrient Management (INM) as a dynamic concept of nutrient management considers the economic yield in terms of fruit yield coupled with quality on one hand, and soil physico-chemical and microbial prospects on other hand as a marker of resistance against the nutrient mining (arises because of failure to strike a balance between annual nutrient demand versus the quantity of nutrients applied). Soils under citrus differ from other cultivated soils,

which remain fallow for 3-6 months every year forcing depletion of SOM (Bhargava, 2002). In contrast, biological oxidation of existing C continues in soil covered under citrus (Srivastava *et al.*, 2002). Multiple nutrient deficiencies are considered to have a triggering effect on potential source of atmospheric CO_2. Soil carbon stock is hence, considered as an important criterion to determine the impact of INM in the longer version of impact assessment (He *et al.*, 1997). The amount of accumulated C within the rhizosphere soil does not continue to increase with time with increasing C outputs. An upper limit of C saturation level occurs, which governs the ultimate limit of soil C sink and rate of C sequestration in mineral soils, independent of C input rate. An understanding of the mechanism involved in C stabilization in soils is needed for controlling and enhancing soil C sequestration (Goh, 2004) under varying modes of nutrient management. In this background, studies were carried out with three major objectives: (i) response of INM on canopy growth, fruit yield and quality indices, (ii) soil carbon dynamics related fertility changes in response to INM and (iii) INM-induced changes in plant nutrition.

MATERIALS AND METHODS

Development of microbial consortium

Growth promoting microbes were isolated from rhizosphere (0-20 cm) for development of microbial consortium through extensive soil sampling at the experimental site. The microbial diversity existing within rhizosphere soil was characterized, and isolated the promising microbes *viz.*, *Azotobacter chroococcum*, *Bacillus mycoids*, *Pseudomonas fluoriscens*, *Bacillus polymyxa* and *Trichoderma harzianum*. Pure culture of these microbes in value added form was developed and prepared a mixture called microbial consortium (MC) having a minimum shelf life of 6 months. The microbial consortium so developed was evaluated under INM mode.

Field experimental setup

An experiment was carried out in 4-year-old Nagpur mandarin (*Citrus reticulata* Blanco) budded on rough lemon rootstock (*Citrus jambhiri* Lush) at the Experimental Farm of National Research Centre for Citrus at Nagpur during 2007-2011. The initial soil fertility analysis showed the variation in different nutrients *viz*., available N 115.6 – 121.5 mg/kg, P 9.4 – 10.2 mg/kg, K 116.7 – 128.7 mg/kg, Fe 7.44 – 8.86 mg/kg, Mn 6.01 – 7.85 mg/kg, Cu 1.10 – 1.75 mg/kg and Zn 0.70-0.78 mg/kg. The soil was taxonomically classified as Vertic Ustochrept. A total of 11 treatments based on recommended dose fertilizers (RDF) *viz*., T_1 - 100% RDF; T_2 - 75% RDF + MC; T_3 - 75% RDF + 25% Farmyard manure (computed on N equivalent basis); T_4 - 50% RDF + 50% Farmyard manure (computed on N equivalent basis); T_5 - 75% RDF + 25% FYM + MC; T_6 - 50% RDF + 50% FYM + MC; T_7 - 75% RDF + 25% vermicompost (computed on N equivalent basis); T_8 - 50% RDF + 50% vermicompost (computed on N equivalent basis); T_9 - 75% RDF + 25% vermicompost + MC; T_{10} - 75% RDF + green manuring (Sunhemp) + MC (added after the cutting at just flower initiation stage) and T_{11} - 50% RDF + green manure (Sunhemp) + MC (added after the cutting, at just flower initiation stage), replicated five times were evaluated in a completely randomized block design. The RDF varied as : 200-600 g N + 50-200 g P_2O_5 + 100-300 g K_2O + 50-200 g Z_nSO_4 + 50-200 g $FeSO_4$ + 50-200 g $MnSO_4$ /tree/year). These fertilizer rates increased with orchard age. Nutrient compositions of different organic manures were 1.20%N, 0.09% P, 0.72%K, 389 ppm Fe, 51 ppm Mn, 30 ppm Cu and 24 ppm Zn in FYM, 2.28% N, 0.11% P, 1.41% K, 3012 ppm Fe, 118 ppm Mn, 34 ppm Cu and 58 ppm Zn in vermicompost, and 1.10% N, 0.08% P, 0.58% K, 280 ppm Fe, 68 ppm Mn, 28 ppm Cu and 24 ppm Zn in green manure of sunhemp.

Leaf sampling and analysis

Six-to-seven month-old-leaves at 2nd, 3rd or 4th leaf positions from non-fruiting terminals covering 10% trees at an height of 1.5-1.8 m from the ground were sampled (Srivastava *et al*., 1999). Leaf samples were thoroughly washed (Chapman, 1964) and ground using a Wiley Grinding machine to obtain homogeneous samples. Tri-acid ($HClO_4$: HNO_3: H_2SO_4 in 2:5:1) digest of leaf samples (Chapman and Pratt, 1961) were subjected to analysis of P by vanado-molybdo-phosphoric yellow color (0.002 N ammonium molybdate + 0.01 N ammonium metavanadate) method, K flame photometrically, and micronutrients (Fe, Mn, Cu, and Zn) by atomic absorption spectrophotometer (Model GBC-908). While N concentration in index leaves was directly determined by auto-nitrogen analyser (Model-Perkin Elmer-2410).

Soil sampling and analysis

Soil samples were collected from perimeter of trees, the zone having maximum feeder roots concentration at soil depth of 0-20 cm. The collected soil samples were likewise air dried, ground, and passed through 2-mm sieve, and analysed for available N using alkaline $KMnO_4$ steam distillation (Subbiah and Asiza, 1956), Olsen-P using sodium bicarbonate extraction by shaking 2.5 g soil in 50 mL of 0.5 M $NaHCO_3$ for 30 min. and K extractable in 1 N neutral NH4OAc in 1:2 soil:extractant ratio after shaking for 30 min. (Jackson, 1978), and micronutrients (Fe, Mn, Cu and Zn) in 0.05 M (pH 7.3) DTPA- $CaCl_2$ after shaking

20 g soil and 50 mL extractant together for 2 hrs. (Lindsay and Norvell, 1978). The soil organic carbon was estimated following wet digestion method of Walkely and Black (1934).

The soil samples were also analysed for soil microbial population (SMP) in the form of fungal count (FC) and bacterial count (BC) using potato dextrose agar and nutrient agar medium, respectively, enumerated by soil dilution plate method (Wollum, 1982). Similarly, the same soil samples were evaluated for Soil Microbial Biomass Nutrients (SMBN) as microbial carbon (C_{mic}), microbial nitrogen (N_{mic}), and microbial phosphorus (P_{mic}). The equivalent of 20 g of soil was placed into a 120 ml cup and adjusted to 30% moisture to a dry weight base. Samples were either fumigated in $CHCl_3$ under vacuum in the dark at 25 °C for 24 h or were not fumigated. After this period, chloroform contained in the soil was removed by repeated evacuation. Fumigated soil was inoculated with 1 g of moist unfumigated soil and mixed well. A 5 ml vial containing 1 ml of 2 N NaOH was placed in each beaker containing a soil sample. The beakers were sealed and then incubated for 10 days at 25 °C. Soil microbial biomass C was calculated as the difference between C content of fumigated and unfumigated samples with a mineralization constant (*kc*) of 0.44 (Jenkinson, 1988). The similar chloroform extraction method was adopted for N_{mic} with mineralization constant (*kn*) of 0.54. While, P_{mic} was measured as per procedure described by Brookes *et al*. (1982) using mineralization constant (*kp*) of 0.40.

Fruit yield and quality

The data on fruit yield was generated by multiplying average weight of fruit with number of fruits in order to arrive at fruit yield as kg/tree. Different fruit quality parameters *viz*., juice content was calculated as the percentage of whole fruit weight, total soluble solids (TSS) by hand refractometer and acidity titrimetric method (Ranganna, 1986).

RESULTS AND DISCUSSION

Data were generated on biometric response (tree spread and height), fruit yield, quality (TSS, acidity and juice), changes in soil fertility (available N, P, K, Fe, Mn, Cu and Zn), soil microbial biomass (Fungal count and bacterial count), soil microbial biomass (Microbial biomass carbon as C_{mic}, microbial biomass nitrogen as N_{mic} and microbial biomass phosphorous as P_{mic}) nutrients, soil carbon partitioning (Organic carbon, inorganic carbon, total carbon and soil C:N ratio) and leaf nutrient composition (N, P, K, Fe, Mn, Cu and Zn).

Biometric response

Crop biometrics were significantly influenced by different INM-based treatments in terms of incremental growth of canopy volume **(Table 1)**. Interestingly, with regard to average increase in canopy volume, sole application of inorganic fertilizers (1.54 m^3) was very well overtaken by either FYM-based INM treatments (1.49-1.95 m^3) or by vermicompost-based INM treatments (1.88-2.34 m^3), but

Table 1. Response of Nagpur mandarin growth parameters in response to different INM-based treatments (Pooled data)

Treatments	*Canopy volume (m^3)*	*Net increase over initial (m^3)*	*Fruit yield (kg/tree)*	*Fruit quality parameters (%)*		
				Juice	*TSS*	*Acidity*
T_1 (100% RDF)	4.26 (2.72)	1.54	15.6	46.4	9.2	0.78
T_2 (75% RDF + MC)	4.49 (3.12)	1.37	8.4	47.2	8.9	0.78
T_3 (75% RDF + 25% FYM)	5.17 (3.69)	1.51	9.2	47.8	9.3	0.70
T_4 (50% RDF + 50% FYM)	4.01 (2.52)	1.49	9.8	46.9	9.4	0.71
T_5 (75% RDF + 25% FYM + MC)	5.15 (3.27)	1.88	14.5	46.8	9.2	0.79
T_6 (50% RDF + 50% FYM + MC)	4.68 (2.74)	1.95	12.8	46.9	9.4	0.80
T_7 (75% RDF + 25% Vm)	6.32 (4.44)	1.88	14.4	47.2	9.3	0.74
T_8 (50% RDF + 50% Vm)	5.26 (3.35)	1.91	14.6	48.4	9.6	0.70
T_9 (75% RDF + 25% Vm + MC)	5.77 (3.43)	2.34	18.8	48.8	9.7	0.70
T_{10} (75% RDF + Gm + MC)	4.12 (3.1)	1.32	11.2	47.2	9.2	0.74
T_{11} (50% RDF + Gm + MC)	3.01 (2.09)	0.93	10.6	47.2	9.0	0.74
CD (*P=0.05*)	0.18 (NS)	0.12	-	-	-	-

- Figures in parenthesis indicate initial values of canopy volume
- MC stands for microbial consortium developed by isolating the native microbes from the experimental soil (mixture of *Azotobacter chrococcum*, *Bacillus mycoides*, *Bacillus polymyxa*, *Pseudomonas flouriscens* and *Trichoderma harzanium*)
- FYM,Vm,Gm, and RDF stand for farmyard manure,vermicompost, green manuring, and recommended doses of fertilizers, respectively

not by green manuring with either 75% RDF + MC (1.32 m^3) or with 50% RDF + MC (0.93 m^3). Amongst different INM-based combinations, 75% RDF as inorganic fertilizers was not so effective with MC (1.37 m^3) or even with 50% or 75% RDF combined with 50% or 25% FYM (1.84 m^3 or 1.49 m^3) when compared with 100% RDF, unless all the three components of INM (inorganic fertilizers, organic manures and microbial biofertilizers) are tested conjointly. For example, T_5 (1.88 m^3) and T_6 (1.95 m^3) were superior over either T_3 (1.51 m^3) or T_4 (1.49 m^3). While, T_9 (2.34 m^3) produced significantly higher magnitude of response over either T_7 (1.88 m^3) or T_8 (1.91 m^3). MC when combined with 75% RDF was not as effective as when treatments were supplemented with organic manures, thereby, suggesting further that exclusive use of chemical fertilizers was detrimental to multiplication of microbial population in soil due to absence of mineralizable carbon in soil.

Response on fruit yield and quality

Data on response to fruit yield in the first year is just notional, since fruiting was not uniformly distributed. But, none the less, fruit yield response **(Table 1)** of different treatments displayed equally interesting results commensurating with the responses on incremental growth of canopy volume. Amongst the INM-based treatments, FYM-based treatments $T_3 – T_6$ registered a much lower fruit yield (9.2-12.8 kg/tree) than vermicompost-based treatments (14.4-18.8 kg/tree). While green manuring-based treatments (T_{10}-T_{11}) recorded fruit yield little lower (10.6-11.2 kg/tree) than FYM-based treatments (9.2-14.5 kg/tree).Considering these observations, green manuring and vermicompost treatments, have so far produced the best response, although such studies deserve to be interpreted on long term basis. Inclusion of AM (arbuscular mycorrhiza 500 g/plant) + PSB (phosphate solubilising bacteria 100 g/plant) + Az (*Azospirillum* 100 g/plant) + Th (*Trichoderma harzianum* 100 g/plant) to 75% RDF improved the fruit yield of Kinnow' mandarin on rough lemon at Ludhiana, Punjab (Location 1) and in Khasi' mandarin at Tinsukia, Assam (Location 2) through different INM–based treatments (Medhi *et al*., 2006; Murti *et al*., 2008).

Evaluation of these treatments on fruit quality response revealed similar results. Vermicompost-based treatments (T_7-T_9) produced much favourable response on fruit quality (47.2-48.8% juice and 9.3-9.7% TSS) than FYM-based treatments T_3-T_6 (46.8-47.8% juice and 8.9-9.4% TSS), but much better than T_1 (46.4% juice and 9.2% TSS) supplying inorganic fertilizers. Earlier studies showed highest fruit yield with improved quality with 25 kg FYM with 400 g N – 150 g P – 300 g K /plant in Khasi' mandarin on acid red soils (Ghosh and Besra, 1997), 150 kg FYM – 1500 g N – 440 g P_2O5 – 600 g K_2O /tree in Navel orange (El– Koumey *et al*., 2000), 52 kg FYM – 1.82 kg $(NH_4)_2SO_4$–N /tree in Balady' mandarin (Gamal and Ragab, 2003), and 15 kg neem cake – 800 g N – 300 g P_2O_5 – 600 g K_2O /tree /year in sweet orange (Tiwari *et al*., 1997). Arsenidze and Chanukuvadze (1988) observed maximum yield of 'Satsuma' mandarin for the trees receiving PK Ca + FYM with N was applied as ammonium nitrate at 100 g/tree to 4 to 5 year old tree in Western Georgia. The best fruit yield and quality of Balady' mandarin was obtained with the use of FM (a by–product of sugar industry) at a rate of 120 kg + and inorganic 6.0 kg N /tree (Ebrahiem and Mohamed, 2000).

Soil fertility changes

Changes in available supply of nutrients in soil were evaluated based on both macro- as well as micronutrients. Evaluation of different INM-based treatments demonstrated significant changes on available supply of nutrients in soil **(Table 2)**. On equivalent basis, T_3 (137.63 N – 13.8 P – 155.2 K mg/kg) recorded significantly higher level of macronutrients over T_4 (134.5 N – 1.8 P – 150.4 K mg/kg), but statistically at par with T_1 (134.1 N – 12.9 P – 152.7 K mg/kg). These responses were of much lower magnitude with either exclusive use of inorganic fertilizers as T_1 (134.2 N – 12.9 P – 152.7 K mg/kg) or even when combined with MC as T_2 (128.8 N – 11.0 P – 144.4 K mg/kg). These observations lend strong support to the fact that microbial biofertilizers are not compatible with exclusive chemical fertilizers unless cushioned with some C-source as manures. Comparison of FYM (T_5) or vermicompost (T_9)-based INM treatments revealed significantly higher magnitude of available macronutrients with latter (150.9 N – 16.4 P – 168.3 K mg/kg) over former treatment (143.1 N – 15.1 P – 159.9 K mg/kg). While, replacement of organic manure upto 50% of RDF as T_6 was not so effective (140.4 N – 16.4 P – 168.7 K mg/kg), but significantly better than inorganic fertilizers carrying treatment as T_1 (134.2 N – 12.9.P – 152.7 K mg/kg). On the other hand, green manuring in combination with either 75% RDF + MC as T_{10} (135.8.1 N – 15.6 P – 151.4 K mg/kg) or with 50% RDF + MC as T_{11} (135.9 N – 16.0 P – 150.82 K mg/kg) could not match with any of the treatments including inorganic fertilizers but significantly superior over T_2 (128.8 N – 11.0 P – 144.4 K mg/kg) where MC was used in combination with 75% RDF.

The response on changes in availability of micronutrients was similar to that of macronutrients **(Table 2)**. There was no significant response of different treatments on the availability of Cu. The treatments T_1 100% RDF (8.93 Fe – 6.77Mn – 0.77 Zn mg/kg), T_3 with 75% RDF + 25% FYM (9.11 Fe – 6.91 Mn – 0.72 Zn mg/kg) showed no significant difference between them, while inferior to T_2 carrying 75% RDF + MC (7.84 Fe – 6.11 Mn – 0.72 Zn mg/kg) supporting

Table 2. Response of different INM-based treatments on the available nutrients (mg/kg) in soil (Pooled data)

Treatments	*N*	*P*	*K*	*Fe*	*Mn*	*Cu*	*Zn*
T_1 (100% RDF)	134.2 (119.2)	12.9 (10.2)	152.7 (125.0)	8.93 (8.03)	6.77 (6.20)	1.45 (1.49)	0.77 (0.72)
T_2 (75% RDF + MC)	128.8 (120.1)	11.0 (9.8)	144.4 (124.6)	7.84 (8.50)	6.11 (7.34)	1.56 (1.10)	0.72 (0.76
T_3 (75% RDF + 25% FYM)	137.6 (117.4)	13.8 (9.2)	155.2 (126.2)	9.11 (8.86)	6.91 (6.34)	1.54 (1.51)	0.80 (0.70)
T_4 (50% RDF + 50% FYM)	134.5 (116.2)	11.8 (10.1)	150.4 (119.2)	8.68 (9.37)	6.83 (6.01)	1.48 (1.60)	0.80 (0.72)
T_5 (75% RDF + 25% FYM + MC)	143.1 (118.7)	15.1 (9.7)	159.9 (116.5)	9.87 (7.77)	7.50 (7.60)	1.50 (1.25)	0.91 (0.74)
T_6 (50% RDF + 50% FYM + MC)	140.5 (117.9)	15.0 (10.0)	161.1 (116.7)	9.58 (8.56)	7.61 (7.21)	1.52 (1.49)	0.88 (0.72)
T_7 (75% RDF + 25% Vm)	142.7 (115.6)	14.8 (9.9)	158.5 (122.9)	9.51 (8.38)	7.77 (7.85)	1.58 (1.51)	0.88 (0.75)
T_8 (50% RDF + 50% Vm)	143.1 (117.5)	14.7 (9.6)	159.2 (126.1)	9.48 (7.22)	7.91 (6.87)	1.53 (1.30)	0.87 (0.78)
T_9 (75% RDF + 25% Vm + MC)	150.9 (119.3)	16.4 (9.8)	168.7 (128.7)	11.42 (7.44)	9.38 (7.12)	1.54 (1.26)	0.98 (0.78)
T_{10} (75% RDF + Gm + MC)	135.8 (120.4)	15.6 (9.4)	151.4 (123.6)	9.95 (8.21)	7.74 (7.08)	1.57 (1.18)	0.90 (0.70)
T_{11} (50% RDF + Gm + MC)	135.9 (121.5)	16.0 (9.9)	150.8(122.4)	9.50 (8.14)	7.70 (7.21)	1.55 (1.75)	0.87 (0.71)
CD (*P=0.05*)	2.4	0.70	2.7	0.51	0.41	NS	0.06

- Figures in parenthesis represent the initial values
- MC stands for microbial consortium developed by isolating the native microbes from the experimental soil (mixture of *Azotobacter chrococcum*, *Bacillus mycoides*, *Bacillus polymyxa*, *Pseudomonas flouriscens* and *Trichoderma harzanium*)
- FYM,Vm,Gm, and RDF stand for farmyard manure,vermicompost, green manuring, and recommended doses of fertilizers, respectively.

the earlier observation about the ineffectiveness of MC with inorganic fertilizers.

While green manuring supplemented with 75% RDF (9.95 Fe – 7.74 Mn – 0.90 Zn mg/kg) or 50% RDF (9.50 Fe – 7.70 Mn – 0.87 Zn mg/kg) with MC was not as effective compared to either FYM or vermicompost-based INM treatments. On the other hand, effectiveness of MC was more visible when treatments carried both organic manures as well as inorganic fertilizers, e.g., T_5 (9.87 Fe – 7.50 Mn – 0.91 Zn mg/kg) was superior over T_3 (9.11 Fe – 6.91 Mn – 0.80 Zn mg/kg) or T_6 (9.58 Fe – 7.61 Mn – 0.88 Zn mg/kg) being superior over T_4 (8.68 Fe – 6.83 Mn – 0.80 Zn mg/kg). Similarly, amongst vermicompost-based treatments, T_9 (11.42 Fe – 9.38 Mn – 0.98 Zn mg/kg) proved better than T_7 (9.51 Fe – 7.77 Mn – 0.88 Zn mg/kg). The combination of 50% RDF + 50% organic manure, vermicompost at 50% produce higher supply of available Mn (7.91 mg/kg) than FYM (6.83 mg/kg) with other micronutrients remaining statistically unchanged.

Soil microbial population

An increase in the microbial biomass often goes along with increased nutrient immobilisation. Over the years, the concepts of INM and integrated soil management (ISM) have been gaining acceptance, moving away from a more sectoral– and inputs–driven approach. INM advocates the careful management of nutrient stocks and flows in a way that leads to profitable and sustained production. ISM emphasises the management of nutrient flows, but also highlights other important aspects of soil complex such as maintaining organic matter content, soil structure, moisture, and microbial biodiversity. Still more attention is needed towards integrated soil biological management as a crucial aspect of soil fertility management, since providing protection to citrus rhizosphere against the nutrient depletion is of utmost importance for sustained orchard production in which the objectivity of INM could have far reaching consequences.

In our studies, the soil microbial count in terms of bacterial and fungal count showed significant changes in response to different INM-based treatments **(Table 3)**. Bacterial and fungal counts both reduced to 31 and 16 x 103 cfu/g, respectively, with T_2 incorporating MC with 75% RDF from corresponding values of 32 and 16 x 103 cfu/g with T_1 having 100% RDF as inorganic fertilizers supporting the fact that as long as good soil productivity is obtained, soil microbial health could be maintained even with inorganic fertilizers. Of course, the magnitude of such response will be of comparatively lower order when compared with organic manures or in combination with inorganic fertilizers plus microbial biofertilizers. Out of two organic manures (FYM versus vermicompost), vermicompost-based treatments produced much favourable response on soil microbial counts as evident from superiority of T_7 (50 and 25 cfu /g bacterial and fungal counts, respectively) over T_3 (45 and 19 x 103 cfu /g bacterial and fungal counts, respectively). Similarly, treatment T_8 (53 and 26 x 103 cfu /g bacterial and fungal counts, respectively) was better than T_4 (44 and 20 x 103 cfu /g bacterial and fungal counts, respectively). Likewise with MC also, vermicompost-based INM module as T_9 (68 and 41 x 103 cfu /g bacterial and fungal counts, respectively) supporting much better soil microbial counts compared to FYM-based INM as T_5 (57 and 25 x 103 cfu /g bacterial and fungal counts,

respectively) and T_6 (48 and 26 x 103 cfu /g bacterial and fungal counts, respectively). On the contrary, inclusion of green manuring with 75% RDF plus MC (50 and 25 x 103 cfu /g bacterial and fungal counts, respectively, with T_{10}) and 50% RDF + MC (44 and 25 x 103 cfg /g soil bacterial and fungal counts, respectively, with T_{11}), although brought some favourable changes on soil microbial counts, but were more significant of lower order when compared to other FYM or vermicompost-based INM treatments (e.g., T_1, T_6, T_9 etc.).

changes were still of lower order in the absence of microbial consortium treatments. The treatments like T_5 (169.7 mg/kg C_{mic}, 29.6 mg/kg N_{mic} and 17.6 mg/kg P_{mic}), T_6 (169.2 mg/kg C_{mic}, 30.3 mg/kg N_{mic} and 19.3 mg/kg P_{mic}) and T_9 (202.5 mg/kg C_{mic}, 49.4 mg/kg N_{mic} and 24.5 mg/kg P_{mic}) produced the best response compared to treatments with MC (e.g., T_3, T_4, T_7 and T_8). These responses supported the fact that all the three components of INM are mandatory in order to harness the best effectiveness of different INM modules.

Table 3. Changes in total bacterial and fungal count of soil samples (0-15 cm) in response to different INM-based treatments (Pooled data)

Treatments	*SMB ((cfu x 103 / g soil)*		*SMBN (mg/kg)*		
	Bacterial count	*Fungal count*	*Cmic*	*Nmic*	*Pmic*
T_1 (100% RDF)	32	16.3	152.1	19.1	16.1
T_2 (75% RDF + MC)	31	16	146.3	19.3	15.2
T_3 (75% RDF + 25% FYM)	45	19	159.1	23.9	15.9
T_4 (50% RDF + 50% FYM)	44	20	164.1	25.6	17.0
T_5 (75% RDF + 25% FYM + MC)	57	25	169.7	29.6	17.6
T_6 (50% RDF + 50% FYM + MC)	48	26	169.2	30.3	19.3
T_7 (75% RDF + 25% Vm)	50	25	169.6	29.2	18.9
T_8 (50% RDF + 50% Vm)	53	26	176.1	29.8	20.4
T_9 (75% RDF + 25% Vm + MC)	68	41	202.5	49.4	24.5
T_{10} (75% RDF + Gm + MC)	50	25	178.6	31.8	18.6
T_{11} (50% RDF + Gm + MC)	44	25	170.7	28.6	17.2
CD (*P = 0.05*)	3.7	2.2	2.6	1.9	3.0

- MC stands for microbial consortium developed by isolating the native microbes from the experimental soil (mixture of *Azotobacter chrococcum*, *Bacillus mycoides*, *Bacillus polymyxa*, *Pseudomonas flouriscens* and *Trichoderma harzanium*)
- FYM,Vm,Gm, and RDF stand for farmyard manure,vermicompost, green manuring, and recommended doses of fertilizers, respectively
- SMB and SMBN stand for soil microbial population and soil microbial biomass nutrients, respectively

Microbial biomass nutrients

Rhizopshere microbial environment modification through roof exudation is an important attribute that regulates not only the availability of nutrient soil, but also their acquisition plants. Different INM-based treatments **(Table 3)** were accompanied with significant changes in soil microbial biomass nutrients in terms of microbial carbon (C_{mic}), microbial N (N_{mic}) and microbial P (P_{mic}). Combination of 75% RDF + MC as T_2 brought down the soil microbial biomass nutrients (146.3 mg/kg C_{mic}, 19.3 mg/kg N_{mic} and 15.2 mg/kg P_{mic}) compared to 100% RDF as T_1 (152.1 mg/kg C_{mic}, 19.1 mg/kg N_{mic} and 16.1 mg/kg P_{mic}). But incorporation of organic manure either FYM (159.1 mg/kg C_{mic} 23.9 mg/kg N_{mic} and 15.9 mg/kg P_{mic} with T_3 or 164.1 mg/kg C_{mic}, 25.6 mg/kg N_{mic} and 17.0 mg/kg with T_4) or vermicompost (169.6 mg/kg C_{mic}, 29.2 mg/kg N_{mic} and 18.9 mg/kg P_{mic} with T_7 or 176.1 mg/kg Cmic, 29.8 mg/kg N_{mic} and 20.4 mg/kg P_{mic} with T_8) helped further in recording higher level of soil microbial biomass nutrients. But these

Soil carbon loading

The treatments evaluated for changes in soil carbon stock (soil organic carbon, soil inorganic carbon as $CaCO_3$ and total carbon) showed significant changes **(Table 4)** which eventually affected soil C:N ratio to varying proportions. The highest level of soil carbon fractions (7.48 g/kg organic carbon,1.88 g/kg inorganic carbon and 9.36 g/kg total carbon) was observed with T_9 followed by T_8 (6.96 g/kg organic carbon, 2.06 g/kg inorganic carbon and 9.02 g/kg total carbon). All these treatments were vermicompost based. While on equivalent basis, different FYM-based treatments like T_5 (6.77 g/kg organic carbon, 2.05 g/kg inorganic carbon and 8.82 g/kg total carbon), T_6 (6.86 g/kg organic carbon, 2.05 g/kg inorganic carbon and 8.91 g/kg total carbon) and T_4 (6.44 g/kg organic carbon, 2.09 g/kg inorganic carbon and 8.53 g/kg total carbon) were not so effective and significantly inferior to vermicompost-based treatments. The treatments T_{10} (6.61 g/kg organic carbon, 1.92 g/kg inorganic carbon and 8.53 g/kg total carbon) and

T_{11} (6.44 g/kg organic carbon, 1.94 g/kg inorganic carbon and 8.38 g/kg total carbon) comprising green manuring with 75% and 50% RDF + MC recorded significant reduction in soil carbon fractions compared to T_1 where exclusive use of inorganic fertilizers were tested. Decimal change in soil C: N ratio was commonly observed. The soil C:N ratio varied from lowest level of 12.7 with T_9 to highest level of 13.9-14.4 with T_3, T_4, T_5, and T_7, with T_1 (having exclusive level of inorganic fertilizers) registering C:N ratio of 14.0. Green manuring-based INM treatments like T_{10} and T_{11} registered C: N ratio of 12.6-12.7:1.

to T_3 (2.13% N – 0.092% P – 1.22% K – 30.8 ppm Fe – 28.2 ppm Mn – 19.6 ppm Zn). Likewise another FYM-based INM treatment, T_6 (2.23% N – 0.0113% P – 1.33% K – 33.5 ppm Fe – 31.2 ppm Mn – 20.6 ppm Zn) was superior to T_4 without MC treatment (2.15% N – 0.092% - 1.24% K – 31.4 ppm Fe – 29.4 ppm Mn – 20.1 ppm Zn). These observations suggested that unless any treatment possess all the three components of INM, it cannot be so effective.

All the vermicompost-based INM treatments demonstrated much superior response on leaf nutrient composition over

Table 4. Response of different INM-based treatments on soil carbon loading (Pooled data)

Treatments	*Soil carbon fractions (g/kg)*			*Soil*	*Soil C:N*
	Organic carbon	*Inorganic carbon*	*Total carbon*	*total N (g/kg)*	*Ratio*
T_1 (100% RDF)	6.33	2.05	8.38	0.597	14.0:1
T_2 (75% RDF + MC)	6.14	2.04	8.18	0.575	14.2:1
T_3 (75% RDF + 25% FYM)	6.61	2.09	8.70	0.604	14.4:1
T_4 (50% RDF + 50% FYM)	6.44	2.09	8.53	0.613	13.9:1
T_5 (75% RDF + 25% FYM + MC)	6.77	2.05	8.82	0.640	13.8:1
T_6 (50% RDF + 50% FYM + MC)	6.86	2.05	8.91	0.650	13.7:1
T_7 (75% RDF + 25% Vm)	6.97	2.02	8.99	0.634	14.1:1
T_8 (50% RDF + 50% Vm)	6.96	2.06	9.02	0.649	13.9:1
T_9 (75% RDF + 25% Vm + MC)	7.48	1.88	9.36	0.735	12.7:1
T_{10} (75% RDF + Gm + MC)	6.61	1.92	8.53	0.676	12.6:1
T_{11} (50% RDF + Gm + MC)	6.44	1.94	8.38	0.660	12.7:1
CD (*P = 0.05*)	0.11	0.4	0.13	0.044	-

- MC stands for microbial consortium developed by isolating the native microbes from the experimental soil (mixture of *Azotobacter chrococcum*, *Bacillus mycoides*, *Bacillus polymyxa*, *Pseudomonas flouriscens* and *Trichoderma harzanium*)
- FYM,Vm,Gm, and RDF stand for farmyard manure,vermicompost, green manuring, and recommended doses of fertilizers, respectively

Leaf nutrient composition

The concentration of both macro-(NPK) and micronutrients (Fe, Mn, Zn) except Cu was significantly influenced by various INM-based treatments **(Table 5)**. Compared to exclusive use of chemical fertilizers as T_1 carrying 100% RDF (2.13% N – 0.088% P – 1.23% K – 30.3 ppm Fe – 28.4 ppm Mn – 19.2 ppm Zn), the treatment T_2 where 25% RDF was replaced by MC (2.08% N – 0.085% P – 1.16% K – 28.7 ppm Fe – 26.8 ppm Mn, - 18.6 ppm Zn) and T_3 replacing 25% RDF with FYM (2.13% N – 0.092 % P – 1.22% K – 30.8 ppm Fe, 28.2 ppm Mn 19.6 ppm Zn) were not statistically superior. Even replacement of 50% RDF through T_4 (2.15% N – 0.092% P – 1.24% K – 31.3 ppm Fe – 29.4 ppm Mn – 20.1 ppm Zn) was neither better to 100% RDF as T_1.

However, incorporation of MC brought some encouraging changes in leaf nutrient composition. For example, treatment T_5 (2.21% N – 0.0116% P – 1.32% K – 33.2 ppm Fe - 30.8 ppm Mn – 20.9 ppm Zn) was significantly superior

FYM-based treatments **(Table 5)**. The treatment T_9 (2.28 N – 0.125% P – 1.42% K 36.4 ppm Fe – 32.8 ppm Mn – 22.6 ppm Zn) registered much higher concentration of all the nutrients compared to T_7 without MC (2.20% N – 0.101% P – 1.31% K – 32.8 ppm Fe – 30.3 ppm Mn – 20.4 ppm Zn) or even T_8 (2.21% N – 0.074% P – 1.29%K – 33.2 ppm Fe – 30.5 ppm Mn – 21.4 ppm Zn). On the other hand, green manuring-based treatments like T_{10} (2.16% N – 0.095% P – 1.30% K – 32.4 ppm Fe – 30.0 ppm Mn – 20.7 ppm Zn) and T_{11} (2.13% N – 0.092% P – 1.28% K – 32.9 ppm Fe – 30.1 ppm Mn – 21.2 ppm Zn) showed statistically compatible effect on leaf nutrient composition compared to treatments comprising either T_1 or T_2 **(Table 5)**. A number of studies indicated improved efficacy of fertilizer nutrients with combined use of manures and fertilizers (Prasad and Singhania, 1989; Rokba *et al.*, 1995) or combined use of inorganic fertilizers (Singh *et al.*, 1993; Medhi *et al.*, 2006) in addition to enzymatic activities (acid phosphatase, dehydrogenase, and urease) of soil (Tiwari, 1996).

Table 5. Response of different INM-based treatments on leaf macronutrients concentration (Pooled data)

Treatments	*N*	*P*	*K*	*Fe*	*Mn*	*Cu*	*Zn*
		(%)			(*ppm*)		
T_1 (100% RDF)	2.13 (1.98)	0.088(0.088)	1.23(1.10)	30.3(24.1)	28.4(23.0)	7.3(6.8)	19.2(16.2)
T_2 (75% RDF + MC)	2.08(1.92)	0.085(0.084)	1.16(1.10)	28.7(28.6)	26.5(26.1)	7.4(5.9)	18.6(17.4)
T_3 (75% RDF + 25% FYM)	2.13(1.98)	0.092(0.082)	1.22(1.12)	30.8(26.0)	28.2(27.2)	7.3(7.1)	19.6(16.3)
T_4 (50% RDF + 50% FYM)	2.15(1.96)	0.092(0.084)	1.24(1.00)	31.4(30.3)	29.4(24.1)	7.0(6.7)	20.1(16.6)
T_5 (75% RDF + 25% FYM + MC)	2.21(1.96)	0.116(0.084)	1.32(1.10)	33.2(31.1)	30.8(23.4)	6.9(6.5)	20.9(17.3)
T_6 (50% RDF + 50% FYM + MC)	2.23(1.98)	0.113(0.084)	1.33(1.06)	33.5(32.3)	31.2(24.1)	7.0(7.1)	20.6(18.3)
T_7 (75% RDF + 25% Vm)	2.20(1.94)	0.101(0.088)	1.31(1.08)	32.8(32.8)	30.3(22.7)	6.9(6.2)	20.4(17.8)
T_8 (50% RDF + 50% Vm)	2.21(1.96)	0.074(0.089)	1.29(1.20)	33.2(30.5)	30.5(24.3)	6.8(7.3)	21.4(16.9)
T_9 (75% RDF + 25% Vm + MC)	2.28(1.94)	0.125(0.078)	1.42(1.12)	36.4(32.0)	32.8(21.9)	7.3(6.4)	22.6(17.2)
T_{10} (75% RDF + Gm + MC)	2.16(1.90)	0.095(0.084)	1.30(1.18)	32.4(25.1)	30.0(25.4)	7.2(7.0)	20.7(17.4)
T_{11} (50% RDF + Gm + MC)	2.13(1.94)	0.092(0.086)	1.28(1.18)	32.9(23.4)	30.1(23.6)	6.9(6.1)	21.2(17.6)
CD (*P=0.05*)	0.06	0.004	0.083	1.17(NS)	1.25	NS	0.63(NS)

- Figures in parenthesis represent the initial values
- MC stands for microbial consortium developed by isolating the native microbes from the experimental soil (mixture of *Azotobacter chrococcum*, *Bacillus mycoides*, *Bacillus polymyxa*, *Pseudomonas flouriscens* and *Trichoderma harzanium*)
- FYM,Vm,Gm, and RDF stand for farmyard manure,vermicompost, green manuring, and recommended doses of fertilizers, respectively.

The above results have ably demonstrated that the ultimate rationale of INM is the judicious use of its all the three principal components *viz*., exploiting the existing synergism between dual purpose microbe (growth promoting as well as biocontrol agent against soil–borne pathogens) types with limited use of inorganic chemical fertilizers, triggering the multiplication of indigenous soil microbial diversity through a suitable substrate of organic origin, in such a way that the nutrients inflow always exceeds the nutrients leaving the system, besides ensuring the market favouring production economics (Srivastava and Ngullie, 2009). However, still there are many core areas where an urgent redressal is required in order to tag INM, a globally vibrant nutrient management strategy to sustain long term quality production and guard against any possible nutrient mining.

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Evaluation of compatibility and effect of *Trichoderma harzianum* and *Pseudomonas fluorescens* against disease complex of tomato

R. Manoj Kumar, M.S. Rao, J. Gavaskar and D.S. Sowmya

Division of Entomology and Nematology, Indian Institute of Horticultural Research, Bangalore – 560 089, India
Corresponding author: msraobio45@gmail.com, msrao@iihr.ernet.in

ABSTRACT

The antagonistic fungal species *Trichoderma harzianum* (teleomorph: *Hypocrea lixii*) is a versatile bio-control agent and is used for the management of pathogenic fungi infecting various crops. Some strains are also effective against nematodes. For the effective management of disease complex of crops caused by nematode + fungus/ bacterium, it is often necessary to use a combination of two or more bio-agents. This necessitates studies on the compatibility of the strains of bio-agents to be applied together. Hence, five strains of *T. harzianum* (Th1-Th5) were evaluated for their compatibility with two strains of *Pseudomonas fluorescens* Migula (Pf1 and Pf2) *in vitro* by direct confrontation assays and *in vivo* in tomato by means of root colonization/rhizospheric competency. The results indicated that different isolates of *T. harzianum* are compatible with *P. fluorescens* both *in vitro* and *in vivo*. Each of the strains showed different levels of compatibility. The strains with higher compatibility of 89.1% have shown a better performance in the field in reducing the nematode induced disease complex caused by *Meloidogyne incognita* and *Fusarium oxysporum* f.sp. *lycopersici*. A reduction in soil nematode population by 52.7% and reduction in percent disease incidence by 61.4% was observed. The results suggest that the combined application of the highly compatible strains of *T. harzianum* and *P. fluorescens*, as seed and substrate treatment, appears promising for the control of nematode induced disease complex under field conditions with a 23.8% increase in yield in tomato.

Keywords: Bio-control agent, compatibility, *Solanum lycopersicum*, tomato

INTRODUCTION

Trichoderma harzianum Rifai (teleomorph: *Hypocrea lixii*) is ubiquitous in most agricultural soils and is a promising antagonist of plant pathogenic fungi and nematodes (Elad *et al*., 1982; Dos Santos *et al*., 1992). It is active as a hyper parasite and has been extensively tested in field experiments. It has been shown to be an effective bio-control agent, with good *in vitro* antagonistic abilities against a range of economically important aerial and soil-borne fungal plant pathogens (Gao *et al*., 2002; Howell, 2003; Harman *et al*., 2004; 2006; Chen *et al*., 2009). The antagonistic mode of action of the fungus has been shown to be due to the production of antibiotics (Claydon *et al*., 1987; Schirmbock *et al*., 1994) and fungal cell wall degrading enzymes such as chitinases, glucanases and proteases (Chet, 1987; Lorito *et al*., 1993). The endochitinase of *T. harzianum* has been shown to be the most effective enzyme when tested alone or in combination with β-1,3-glucanase. It appears to be more effective than plant and bacterial chitinases against a wide range of target fungi (Lorito *et al*., 1993, 1994).

Siddiqui *et al*. (1999) reported the effect of a combination of *P. aeruginosa* Migula and *T. viride* Pers. in the control of root rot and root-knot disease complex in chilli. Siddiqui and Shaukat (2003) demonstrated that the non-pathogenic *Fusarium solani* strain Fs5 substantially increased the bio-control performance of the DAPG-producing *P. fluorescens* strain CHA0 in tomato.

Perusal of the literature clearly indicates the need to generate data on the compatibility of *T. harzianum* and *P. fluorescens*. In the past several local isolates of these bio-agents were tested for their efficacy in controlling root-knot and other nematodes (Rao *et al.,* 1997; 1998a; 2003; 2004; Rao and Dhananjay, 2003; Rao and Shylaja, 2004; Rao 2007; 2008). Siddiqui and Shaukat, 2004 reported that certain strains of *T. harzianum* enhances the production of nematicidal compounds *in vitro* and improve biocontrol of nematodes by *P. fluorescens*. Hence, in this work five strains of *T. harzianum* were evaluated *in vitro* for their compatibility with two strains of *P. fluorescens* by direct confrontation of dual culture method. The evaluation of the compatibility, in terms of rhizospheric competency, was also performed *in vivo*, in tomato under glass-house conditions.

MATERIALS AND METHODS

Compatibility of *T. harzianum* and *P. fluorescens in vitro*

Direct confrontation experiment by dual culture method was adopted to evaluate the compatibility of strains/isolates of *T. harzianum* (Th1-Th5) and *P. fluorescens* (Pf1 and Pf2) *in vitro*. The strains tested are maintained in collection of the Division of Entomology and Nematology, Indian Institute of Horticultural Research, Bangalore, India by cryopreservation method as described by Sudheer (2010). The strains of *P. fluorescens* (Pf1 and Pf2) were selected because they were reported to be efficient antagonists of nematodes (Rao *et al*., 2004; Rao and Shylaja, 2004; Rao, 2007, 2008). The strains of *T. harzianum* Th1-Th5 were selected out of 48 local isolates based on the presence of PRA 1 gene. PRA1 was the major responsible gene reported only in strains of *T. harzianum* exhibiting antagonism against nematodes (Suarez *et al*., 2004). These strains were isolated on *Trichoderma* selective media from the soil samples collected from undisturbed ecosystems nearby Bangalore and Shimoga regions from Karnataka state, India by the method described by Papavizas and Lumsden (1982) and Mukherjee (1991) and used in the experiment after molecular screening for the presence of the PRA1 gene (Manoj Kumar, 2011).

Fresh cultures of *P. fluorescens* were prepared in 100 ml Erlenmeyer flask containing Kings'B broth (King *et al*., 1954). A loopful of *P. fluorescens* culture was inoculated 24 hrs before the start of the experiment, incubated at 37 °C on a rotating shaker incubator at 150 RPM and used for the experiments. Fresh cultures of *T. harzianum* were prepared by inoculating 5 mm culture disc of *T. harzianum* made by using a cork borer. The discs were inoculated on potato dextrose agar medium solidified plates and incubated at 27 ± 2 °C for 5 days to obtain fresh cultures. Five mm diameter circular culture discs of strains (Th1-Th5) were punched at the periphery of the fungal growth in the Petri dishes and used in the direct confrontation assay. Petri dishes (90 mm) plated with potato dextrose agar were used in the experiments. In all plates, *P. fluorescens* was inoculated 6 hrs earlier than *T. harzianum*. Culture discs of *T. harzianum* were inoculated 20 mm from the periphery of the plate. Different combinations of various strains/ isolates of *T. harzianum* (Th1 – Th5) and *P. fluorescens* (Pf1 and Pf2) **(Table 1)** were evaluated separately. There were six replicated plates per treatment, incubated at 27 ± 1 °C. Observations were recorded on the compatibility interactions *in vitro* for 7 days.

The compatibility of *P. fluorescens* and *T. harzianum* was evaluated in a dual culture test as described by Abeysinghe (2009), Barret *et al*. (2009) and Chariklia *et al*. (2011). *P. fluorescens* strain was streaked (zigzag) 25 mm from the edge of a Petri plate (90 mm diameter) containing potato dextrose agar, and allowed to grow for 48 h. A disc (5 mm diameter) taken from the edge of an actively growing colony of *T. harzianum* strain on potato dextrose agar was then placed in Petri plate at 25 mm from the periphery of Petri plate and facing *P. fluorescens* and the plates were sealed with parafilm. The same was repeated for combinations of strains/isolates of *T. harzianum* (Th1-Th5) and *P. fluorescens* (Pf1 and Pf2). Petri dishes inoculated only with *T. harzianum* (strains Th1-Th5) separately, without inoculation of *P. fluorescens*, were used as controls (Fig. 1). Each combination was tested for its compatibility by using the same dual culture method **(Table 1)**. Each treatment had six replicates (6 plates). The plates were incubated in a BOD for six days at 27 ± 2 °C. Observations were recorded after every 24 hrs on the diameter growth of *T. harzianum* (strains Th1-Th5) in the presence of *P. fluorescens* strains. The diameter of the fungal colony in each Petri plate was recorded by taking the average of two measurements at right angles per plate. Compatibility was calculated from the measurements recorded on the 6th day. The experiment was repeated thrice.

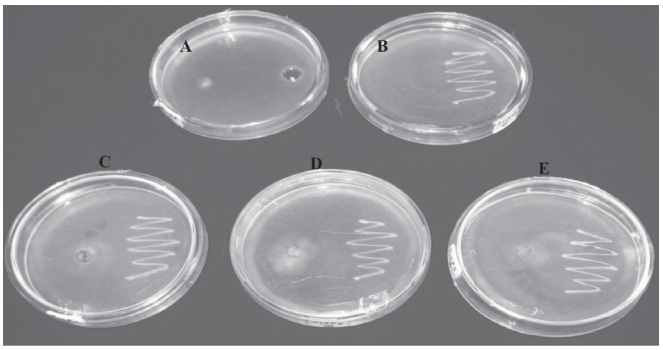

Fig. 1. Picture showing the procedure followed in the dual culture method by direct confrontation assay.

Percent compatibility was calculated by using the formula:

$$\text{compatibility (\%)} = 100 - \%\ 'I'$$

where I is % Inhibition or Increase.

The per cent inhibition in the colony diameter was calculated using the formula (Vincent, 1947):

$$\text{inhibition (\%)} = \frac{\text{Colony diameter in the control - Colony diameter in treatment}}{\text{Colony diameter in control}} \times 100$$

whereas the per cent increase in the colony diameter was calculated using the formula:

$$\text{increase } (\%) = \frac{\text{Colony diameter in the treatment - Colony diameter in control}}{\text{Colony diameter in control}} \times 100$$

Table 1. Percent compatibility of strains of *Trichoderma harzianum* (Th1-Th5) and *Pseudomonas fluorescens* (Pf1 and Pf2) *in vitro*, in dual culture method.

Treatment (Strain combination)	*Average % compatibility*
TH 1 + PF 1	81.9 de
TH 2 + PF 1	76.7 bcd
TH 3 + PF 1	89.1 f
TH 4 + PF 1	86.7 ef
TH 5 + PF 1	73.8 bc
TH 1 + PF 2	76.4 bcd
TH 2 + PF 2	71.7 b
TH 3 + PF 2	81.1 de
TH 4 + PF 2	79.1 cd
TH 5 + PF 2	65.1 a
CD at 1%	7.36

All the figures are the average of five replicates of pooled data. Mean followed by the same letter in each column are not significantly different ($P \leq 0.01$) according to Duncan's multiple range test.

Compatibility of *T. harzianum* and *P. fluorescens in vivo*

Tomato was taken as target crop plant for evaluation of the compatibility of *T. harzianum* and *P. fluorescens* as it is easier to obtain precise data on the root colonization by both bio-agents. The *T. harzianum* strains (Th1-Th5), which proved effective against *Fusarium oxysporum* f.sp. *lycopersici* Snyder *et* Hansen and *M. incognita in vitro* (Manoj Kumar, 2011) were mass produced by inoculating in molasses media (Sankar and Jeyarajan, 1996) and by following the protocol as standardized by Sangeetha *et al*. (1993). Four hundred ml of the mass produced media, 10 g of carboxy methyl cellulose (CMC), along with fungal spores, after 10 days were mixed with 1 kg of sterile talc powder obtained from Mehta and Sons Co., Bangalore, as described by Bhai *et al*. (1994). The CFU/g of these formulations was adjusted at 2×10^6 CFU/g and it was evaluated by taking 1 g of talc formulation using the serial dilution method (Rao *et al*., 1997; Tarun Kumar *et al*., 2005). One ml from each of 10^{-4}, 10^{-5} and 10^{-6} dilutions were pipetted into the Petri dishes and spread completely and plated with potato dextrose agar.

Talc based formulation of *P. fluorescens* (Pf1 and Pf2) was produced by adding 400 ml of 48 hr old bacterial culture in Kings' B medium broth to 1 kg of sterile talc powder. Ten gm of CMC was also added as it helps in binding the bacterial culture to talc powder and then dried as described by Vidhyasekaran and Muthamilan (1995) and Vidhyasekaran *et al*. (1997).

The mass produced *T. harzianum* strains were evaluated for their compatibility with *P. fluorescens* (Pf1 and Pf2) on tomato (cv. Arka vikas) individually and in combination by means of seed treatment and substrate application as described in Rao *et al*. (2004). Seeds were treated with talc based formulation of different strains of *T. harzianum* (2×10^6 CFU/g) or *P. fluorescens* (2×10^8 CFU/g) each at the rate of 10 g/kg of seed. One kg substrate (sterile coco peat made of coconut coir, obtained from Chennakesava Agro Industries, Bangalore) mixed with 5 g of *T. harzianum* alone or in combination with 5g *P. fluorescens* was used to fill each seedling tray (with 98 wells about 10 cm^3 per well).

There were eighteen treatments **(Tables 2&3)**, of which seven treatments were of seed and substrate treated with a formulation containing only one of the seven isolates, ten treatments were of both seed and substrate treated with combinations of each of the five *T. harzianum* isolates with each of the *P. fluorescens* isolates, and a treatment with neither *T. harzianum* nor *P. fluorescens* which served as control. All the experimental plants were maintained in glass house. They were maintained at 35-40 °C by covering seedling trays with black mulching sheet. The plants were watered regularly only in the morning using a watering can. Each treatment was replicated five times in a completely randomized design. The same experiment was repeated thrice.

Estimation of plant growth components: Thirty days after sowing, five tomato seedlings were randomly uprooted from each seedling tray (replicate) and observations were made on length, and weight of fresh shoots (from base of stem to the tip of leaves) and roots (from base of the stem to the bottom of the roots) as described by Rao (2007).

Estimation of root colonization: The seedlings were washed under running distilled water to make it free of cocopeat. They were cut at the base of the stem and the roots were collected, weighed, one gram sample ground and the CFU was estimated by the serial dilution followed by pour plate method. Serial dilutions up to 10^{-6} concentration were prepared. One ml from each of 10^{-4}, 10^{-5} and 10^{-6} dilutions were poured into the Petri dishes and spread completely in the plate. Freshly prepared Trichoderma selective media (Papavizas and Lumsden, 1982) or Kings'B agar media (King *et al*., 1954) was poured into each plate and made to spread evenly by pour plate method and allowed to solidify. Three replicates (3 plates) for each dilution were maintained with controls and CFU was recorded after 24 and 96 hrs for *P. fluorescens* and *T. harzianum*, respectively.

Evaluation of bio-efficacy of compatible strains under field conditions

Vermicompost (100 kg) was enriched by addition of 1 kg

Table 2. Plant growth variables of tomato as affected by the compatibility of combined treatments with strains of *T. harzianum* (Th1-Th5) and *P. fluorescens* (Pf1 and Pf2) *in vivo* and extent of their root colonization.

Treatment	*Seedling length (cm)*	*Seedlin gweight (g)*	*CFU of T. harzianum (× 10⁵/ g root)*	*CFU of P. fluorescens (× 10⁶/ g root)*
TH 1+PF1 - SD+SB	28.9 ij	4.3 fg	2.08 d	1.86 b
TH 2+PF1 - SD+SB	27.1 hij	4.1 fg	2.08 d	1.84 b
TH 3+PF1 - SD+SB	29.4 j	5.4 h	2.12 d	1.98 b
TH 4+PF1 - SD+SB	28.4 ij	4.8 g	2.09 d	1.93 c
TH 5+PF1 - SD+SB	26.2 hij	3.1 e	1.99 c	1.80 b
TH 1+PF2 - SD+SB	24.7 fgh	2.2 cd	1.88 b	1.78 b
TH 2+PF2 - SD+SB	21.4 def	1.9 bc	1.96 b	1.78 b
TH 3+PF2 - SD+SB	25.7 ghi	3.9 f	1.88 b	1.78 b
TH 4+PF2 - SD+SB	24.8 fgh	2.4 cd	1.84 b	1.77 b
TH 5+PF2 - SD+SB	19.3 cde	1.9 bc	2.11 e	2.02 e
TH 1 - SD+SB	18.5 cd	2.2 cd	1.99 d	0.0 a
TH 2 - SD+SB	17.4 c	1.8 bc	2.15 g	0.0 a
TH 3 - SD+SB	20.2 cde	2.7 d	2.05 f	0.0 a
TH 4 - SD+SB	22.3 efg	1.3 ab	1.93 c	0.0 a
TH 5 - SD+SB	20.8 cde	3.1 e	2.27 e	0.0 a
PF1 - SD+SB	13.4 b	2.1 c	0.0 a	2.0 e
PF2 - SD+SB	11.9 b	2.0 c	0.0 a	2.02 e
Control	8.5 a	1.2 a	0.0 a	0.0 a
CD at 5%	3.18	0.60	0.32	0.46

SD = Seed treatment; SB = Substrate treatment.

All the figures are the mean average values of five replicates of pooled data. Mean followed by the same letter in each column are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

formulation of different strains of *T. harzianum* (Th1- Th5) and *P. fluorescens* (Pf1 and Pf2) separately. It was left for 15 days under the shade and optimum moisture content of 25-28% was maintained. The bio-pesticide enriched vermi-compost was added to the field plots of 2 m × 3 m each at the rate of 50 g/m^2 according to the treatments **(Table 3)**. The experiment was conducted at the Indian Institute of Horticultural Research farm in a field infested with *M. incognita* (116±8 per 100 cc of soil) and *F. oxysporum* (2 × 10^5 CFU/g of soil). The texture of the soil in the experimental plot was sandy-clay-loam, with pH of 6.6-6.8 and organic matter of 0.22-0.24%. During the experimental periods of the two seasons, rainfall was of 65 mm (first season) and 73 mm (second season) and soil temperatures were in the range 25-37 °C.

The remaining tomato seedlings (30-day-old) of different treatments were transplanted in the plots amended with enriched vermicompost of respective strains of bio-agents according to the treatments **(Table 3)**. Seedlings derived from non treated seeds or coco-peat and transplanted in plots non amended with enriched vermi-compost served as controls. There were eighteen treatments including control **(Table 3)**. The experiment was repeated for two seasons.

Seedlings were planted with a spacing of 50 × 50 cm in 6 rows (4 plants in each row) and there were five replications per treatment arranged in a randomized block design (RBD). The crop was maintained by applying recommended dosages of fertilizers and plant protection chemicals. Observations of the root-knot index, disease incidence, yield of tomato at harvest (90 days after transplanting), root colonization by *T. harzianum* and *P. fluorescens* were recorded.

Five plants per plot were uprooted at random and the nematode infestation of the roots was estimated according to a 1 to 10 scale suggested by Bridge and Page (1980). Twenty five grams of roots (5 g from each of the five plants per plot) were collected at random. Nematode population in 100 cc soil was estimated by following Cobb's sieving and decanting method (Cobb, 1918) followed by modified Baermann's funnel method (Schindler, 1961). The reductions in the nematode galling index due to the application of different strains of *T. harzianum* or *P. fluorescens* or both were calculated. Further, data on the disease incidence caused by *F. oxysporum* at flowering (the number of plants completely wilted out of total number of plants) was calculated by using the formula.

$$\text{Percent disease incidence (PDI)} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Statistical analysis

Depending upon experimental design, a one-way analysis of variance (ANOVA) was performed using SPSS ver 10.0.

Table 3. Effect of the compatibility of various strains of *T. harzianum* (Th1-Th5) and *P. fluorescens* (Pf1 and Pf2) on the population and root-knot index of *M. incognita*, % disease incidence due to *F. oxysporum* and yield of tomato, 90 days after transplanting.

Treatment	*CFU of T. harzianum (x 10^5)*		*CFU of P. fluorescens (x 10^6)*					
	CFU/g root	*CFU/g soil*	*CFU/g root*	*CFU/g soil*	*Root-knot index (1-10)*	*Nematode population in 100 cc soil*	*% disease incidence due to F. oxysporum*	*% increase in yield*
TH 1+PF1 - SD+SB	3.5 efg	2.6 cde	1.7 bc	2.0 bcd	4.3 c	71 bcde	25.6 bc	17.9 gh
TH 2+PF1 - SD+SB	3.0 def	2.3 bcd	1.5 bc	1.8 bcd	3.9 bc	69.8 bcde	29.2 cd	19.3 i
TH 3+PF1 - SD+SB	4.3 h	3.5 e	2.2 cd	2.3 de	3 a	56.8 a	19.8 a	23.8 j
TH 4+PF1 - SD+SB	3.8 gh	2.9 de	1.4 b	1.8 bcd	4.1 bc	63.2 ab	27.4 cd	17.2 fgh
TH 5+PF1 - SD+SB	3.7 fgh	2.9 de	1.9 bc	2.3 cde	4.1 bc	66.2 bc	27.6 cd	16.7 ef
TH 1+PF2 - SD+SB	3.1 defg	3.0 de	1.5 b	1.9 bcd	4.3 c	76.8 ef	28.2 cd	17.4 fgh
TH 2+PF2 - SD+SB	2.5 bcd	2.0 bc	1.3 b	1.7 bc	3.9 bc	72.2 cde	30.4 cd	18.5 hi
TH 3+PF2 - SD+SB	3.7 fgh	2.6 cde	1.7 bc	2.1 bcd	3.4 ab	68.2 bcd	22.4 ab	19.8 i
TH 4+PF2 - SD+SB	3.1 defg	1.8 bc	1.3 b	1.6 b	4.1 c	65.6 bc	28.8 cd	16.3 def
TH 5+PF2 - SD+SB	2.8 cde	1.6 b	1.4 b	1.6 b	4.4 c	70.6 bcde	30.8 de	15.9 def
TH 1 - SD+SB	2.5 bcd	2.2 bcd	0 a	0 a	5.0 d	82.6 fg	34.2 ef	15.6 de
TH 2 - SD+SB	1.9 b	1.8 bc	0 a	0 a	6.6 e	76.4 def	37.8 fg	15.9 def
TH 3 - SD+SB	2.0 b	1.9 bc	0 a	0 a	5.6 d	73.8 cde	31.8 de	16.1 def
TH 4 - SD+SB	2.3 bc	1.8 bc	0 a	0 a	6.3 e	67.0 bc	32.2 de	14.9 d
TH 5 - SD+SB	2.1 bc	1.9 bc	0 a	0 a	5.9 de	76.4 def	40.6 g	12.8 c
PF1 - SD+SB	0 a	0 a	2.5 de	2.8 ef	6.5 e	82.2 fg	34.8 ef	9.1 b
PF2 - SD+SB	0 a	0 a	2.9 e	3.2 f	6.5 e	86.0 g	40.0 g	9.3 b
Control	0 a	0 a	0 a	0 a	8.1 f	113.8 h	51.4 h	0.0 a
CD – 5%	0.68	0.72	0.50	0.48	0.62	7.1	4.52	1.25

SD = Seed treatment; SB = Substrate treatment followed by application of enriched vermicompost to the experimental plots according to their treatments.

All the figures are the mean average values of five replicates of pooled data. Mean followed by the same letter in each column are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Data of CFUs were log transformed for analysis and re-transformed after analysis (includig CD)

As a follow-up of ANOVA, the treatment means were separated using Fisher's least significant difference (LSD) and Duncan's multiple range tests. Data of CFUs were log transformed for analysis and re-transformed after analysis (includig CD). Data from repeated trials were pooled after confirming the homogeneity of variances.

RESULTS AND DISCUSSION

Compatibility of *T. harzianum* and *P. fluorescens in vitro*

In the past, application of a single bio-agent under field conditions did not prove effective in the management of disease complex caused by the concomitant presence of a nematode and a fungus/bacterium (Faulkner *et al.,* 1970; Taylor, 1990; Rao *et al*., 1998a; 1998b). To combine and use any two organisms in biological control, it is necessary to check their compatibility thoroughly. In our experiments, the level of compatibility varied with the different strains of the bio-agents tested **(Figs 2-6)** and the effect of this variation on the management of the disease complex caused by *M. incognita* and *F. oxysoprum* in tomato was assessed. The overall compatibility was significantly greatest between strains Th3 of *T. harzianum* and Pf-1 of *P. fluorescens* (89.1%) **(Table 1&Fig. 4)**. Hence, the results are useful to standardize the method of application of two or more bio-agents in combination for the effective management of disease complex of crops, as highlighted by different authors (Rao *et al.,* 1997; 1998a; Nagesh and Parvatha Reddy, 2000; Anusuya and Vadivelu, 2002; Manoj Kumar *et al*., 2010).

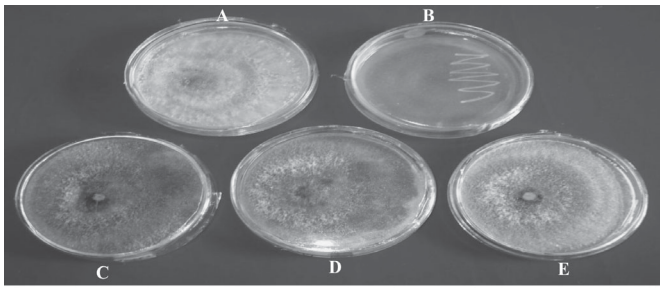

Fig. 2. Compatibility of TH-1 strain of *T. harzianum* and PF-1 strain of *P. fluorescens*.

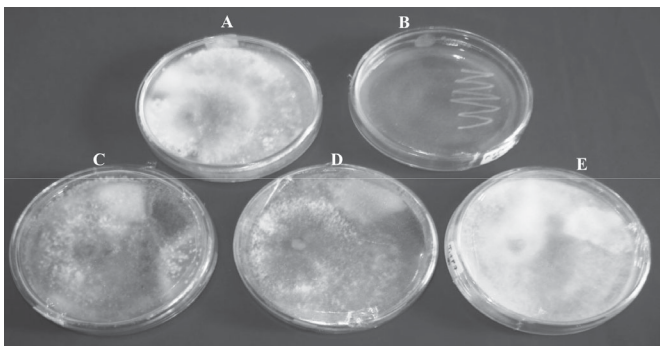

Fig. 3. Compatibility of TH-2 strain of *T. harzianum* and PF-1 strain of *P. fluorescens*.

Evaluation of effect of compatibility of *T. harzianum* and *P. fluorescens in vivo* and in field conditions

The combination treatment of Th3 + Pf1 strains, which

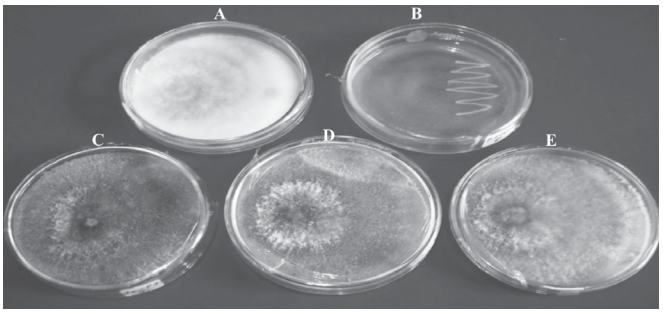

Fig. 4. Compatibility of TH-3 strain of *T. harzianum* and PF-1 strain of *P. fluorescens*

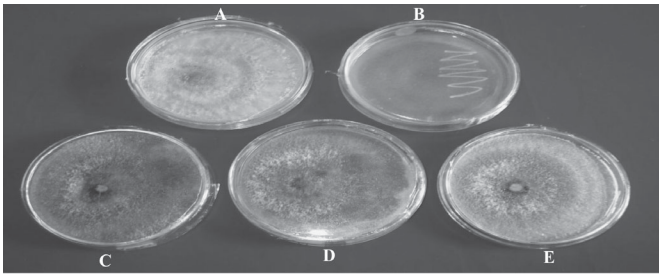

Fig. 5. Compatibility of TH-4 strain of *T. harzianum* and PF-1 strain of *P. fluorescens*.

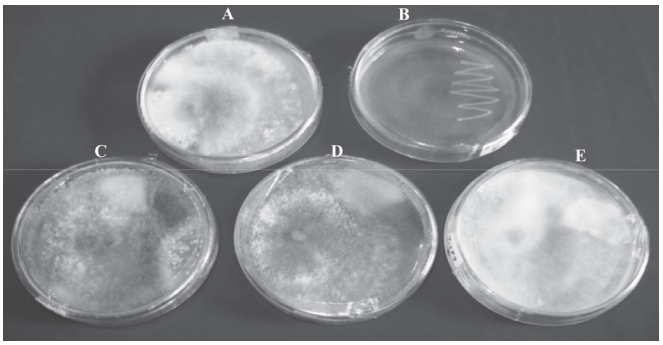

Fig. 6. Compatibility of TH-5 strain of *T. harzianum* and PF-1 strain of *P. fluorescens*.

Note: In Fig. 1 – 6 A- Control plate with *T. harzianum*; B - Control plate with *P. fluorescens*; C, D and E are 3 replicates of the dual culture plates exhibiting compatibility of different strains used.

showed the greatest compatibility *in vitro* **(Table 1)** was found to be the best treatment *in vivo* also as there was significant improvement in plant growth components **(Table 2)**. The increase in the plant growth components treated with the combined formulations of *T. harzianum* and *P. fluorescens* was greatest in treatments involving both substrate and seeds treated with both bio-agents (Th-3 strain of *T. harzianum* and Pf-1 strain of *P. fluorescens*) **(Table 2)**. This contradicts finding by Siddiqui and Shaukat (2004) who reported a significant reduction in plant weight of tomato plants in combination treatments. This finding was in confirmation with findings of Rao, (2007).

There was a general increase in the extent of root colonization by the bio-agents when a combined formulation of both organisms was used to treat seeds and substrate **(Table 2)** and also when transplanted in the field **(Table 3)** after application of enriched vermicompost. *Trichoderma harzianum* did not affect the colonization by *P. fluorescens* and vice versa **(Table 2 & 3)**. The greatest levels of compatibility and rhizospheric competency was observed between the strains Th-3 of *T. harzianum* and Pf-1 of *P. fluorescens in vitro* and *in vivo* as their CFUs increased significantly when used together **(Table 1, 2 & 3)**. Also, when these strains were used together there was an increase in yield and significant reduction in the disease complex caused by *M. incognita* and *F. oxysporum* **(Table 2 & 3)**. Siddiqui and Shaukat (2004) reported that the rhizosphere colonization pattern of two *P. fluorescens* strains, used individually or in combination with *T. harzianum*, did not differ significantly, while it did differ in our experiments **(Table 3)**.

The beneficial effects of the combination treatments of strains of *T. harzianum* and *P. fluorescens* other than Th-3 + Pf-1 were also significant on root galling index, soil nematode population, percent disease incidence and percent yield increase and, with a few exceptions, at par with each other but significantly less than the Th3 + Pf1 combination **(Table 3)**. A significantly lesser performance was observed in the treatments with individual bio-agents compared to the treatments with the combination of various strains of bio-agents. This suggests complementary and synergistic interaction between *T. harzianum* and *P. fluorescens* in the management of the disease complex in tomato leading to increase in yield. With combination treatments, the strains having more compatibility **(Table 1)** resulted also in greater yield increase and lower root galling index and disease incidence **(Table 3)**.

However, in some reports the feasibility of combining *Trichoderma* spp. with fluorescent pseudomonads has been questioned (Hubard *et al*., 1983). According to these authors, indigenous populations of fluorescent pseudomonads significantly reduced the biocontrol activity of *Trichoderma* spp. when applied to control *Pythium* seed rot of pea and iron competition was the primary mechanism involved for this reduction. In contrast, Dandurand and Knudsen (1993) reported that the combination of *P. fluorescens* 2-79 and *T. harzianum* ThzID1 neither inhibited nor enhanced the biocontrol activity of the latter agent against root rot of pea caused by *Aphanomyces euteiches* f. sp. *pisi*. Instead, our findings have shown a reduction in the disease complex in the plants along with increase in the yield **(Table 3)** when *P. fluorescens* and *T. harzianum* were used together compared to single bio-agent treatments. Isolates of *T. harzianum* varying in their compatibility with *P. fluorescens in vitro* **(Table 1)** also varied in their efficacy *in vivo* as they increased root colonization and plant growth components **(Table 2)**. The same variation pattern was observed under field conditions **(Table 3)**. However, this variation was found proportional to the percent compatibility between the strains **(Tables 2 & 3)**. We do not know the reasons for this phenomenon and it is presumed that this could be because of different levels of production of secondary metabolites by both bio-agents when used in combination.

In the present investigation we focused on the possible impact of percent compatibility of different strains of *T. harzianum* with *P. fluorescens in vitro* followed by *in vivo* trials. It was observed that as the CFUs of the bio-agents in the rhizosphere increased in the combination treatments, there was a reduction in root galling index and disease incidence in the plants paired with an increase in yield **(Table 3)**. This did not occur in case of treatments with single bio-agents even though the CFUs in few treatments were at par with some of the combination treatments. This could be due to synergistic effect of strains used in the combination treatment in accordance with finding of Siddiqui and Shaukat (2004) where the presence of *T. harzianum* was reported to enhance the biological control of nematodes by *P. fluorescence* when produced together. These findings may form the basis for the combined application of *T. harzianum* and *P. fluorescens* under the field conditions.

In the field evaluation, the treatment with combination of TH3 +Pf1, showing the greatest compatibility **(Table 1 & 2)**, showed the best performance in reducing nematode infestation, disease incidence and increasing the yield. This performance gradually decreased with the reduction in compatibility of the different strains used in the other combination treatments of *T. harzianum* (Th1- Th5) and *P. fluorescens* (Pf1 and Pf2) **(Table 3)**. When compared with treatments of individual bio-agents of different strains, the treatments with a combination of bio-agents were also

reported to give better performance in previous studies (Rao *et al*., 2004; Rao, 2007, 2008). This proves that the combined use of the highly compatible strains of *T. harzianum* and *P. fluorescens* is more beneficial than that of combined use of lesser compatible strains. However, further research is necessary to obtain more insights on the biochemical aspects of this synergistic effect of compatible strains on plant systems.

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RAPD –based genetic diversity studies and correlation with morphological traits in *Senecio* species of Northern parts of Western Ghats

H. P. Sumangala[1], K. Manivannan[2], A. S. Sidhu[1] and R. Venugopalan[1]

[1]*Indian Institute of Horticultural Research, Bengaluru – 560089*

[2]*Professor and Head, Horticulture Department, Annamalainagar, Chidambaram*

Corresponding author: sumasiddharth@gmail.com, sumangala@iihr.ernet.in

ABSTRACT

Senecio is one of the largest genus belonging to Asteraceae family having great variation and wider adaptability. There are more than 34 species of this particular genus distributed in India. Four *Senecio* species of northern parts of Western Ghats, using RAPD markers and measurements of seven morphological traits *viz.*, plant height, plant spread, days to flowering, number of primary branches, number of secondary branches, number of leaves per plant, number of flowers per plant. PCR amplification with 12 random primers produced 228 bands, observed at 68 amplification loci; 30 percent of the amplified loci were polymorphic, with a mean of 5.6 polymorphic loci per primer. The similarity coefficient ranged from 32 to 39 among the four samples. The minimum genetic similarity has been observed for *S. dalzelli* and *S.belgaumensis.* Cluster analysis resulted in 2 major groups based on geographical distribution that generally reflected expected trends between the genotypes. No correlation was found between RAPD markers and the morphological traits. This study provides information useful for the identification, classification, phylogenies and breeding of *Senecio* species.

Keywords: *Senecio* species, RAPD markers, genetic diversity.

INTRODUCTION

In an era of shrinking natural resources like land and water, importance has been given to the minimum maintenance of garden. Consumers are looking for varieties of plants which require fewer inputs to maintain them. In this context either the native or wild species play a very crucial role in case of landscape gardening. Having met with all different kinds of edaphic factors, India is recognized as one of the twelve mega biodiversity hot spots. There is a tremendous unexploited flowering plant's wealth in our country which needs to be domesticated and there is also a need to create awareness about the floral wealth.

Senecio L. (Asteraceae Bercht. & J. Presl.; Senecioneae Cass.) is one of the largest genera of flowering plants, containing somewhere between 1,000 and 3,000 species (Jeffrey *et al.*, 1977; Nordenstam, 1978; Bremer, 1994; Vincent, 1996; Mabberley, 1997). The most recent and reliable estimate suggests that it comprises approximately 1,250 species (Nordenstam, 2007). The name *Senecio* comes from the Latin "senex" which means "old" (an old man), in reference to the grey pappus (formed from modified calyces) found on the cypsela (the fruit) (Johnson & Smith, 1947).

There are more than 34 species spread across the Western Ghats. Till today, only taxonomical identification has been done about this species in India. The current research sets out to evaluate the wild species of the genus *Senecio* as potential new ornamentals for the floriculture. No information exists regarding the horticultural qualities of either of these species. To determine what experimentation and in-depth evaluation may be required to prepare these species for introduction, the primary information about the diversity would appear to be an example of adaptive radiation, brought about by natural selection favouring types that are adapted to different ecological niches. Information of genetic diversity amongst the adapted cultivars or selected breeding materials has a major impact on the crop improvement. It can be acquired from the pedigree analysis, morphological characters or using molecular markers (Pejic *et al.*, 1998).

Molecular markers are very rapidly being adopted by the researchers all over the world for the crop improvement and are the appropriate and valuable tools for basic and applied studies dealing with the biological mechanism in agricultural production systems (Jones *et al*., 1997). RAPD markers can be of great importance as a fast process for taxonomic studies, (Oliveira *et al*., 1999). Williams *et al*.,

(1990) introduced the random amplified polymorphic DNA (RAPD) method. This technique has efficiently been used for genome mapping in plants (Staub *et al*., 1996) and for the detection of plant disease resistance genes (Martin *et al*., 2003). Random amplified polymorphic DNA (RAPD) markers can be used for the detection of DNA polymorphism without the requirement for predetermined genetic data. Though the selection of breeding material on the basis of morphological characteristics has been an effective method, morphological comparisons may have limitations, including the influence of the environment or management practices (Gepts 1993; Nemera *et al*., 2006). There are various factors which influence the reproducibility of RAPD amplification profiles such as any difference in the process used for DNA isolation (Korbin *et al*., 2000), concentration of primer or Taq-DNA polymerase, annealing temperature, number of thermal cycles and concentration of $MgCl_2$ (Bassam *et al*., 1992; Kernodle *et al*., 1993), template quality and quantity, primer sequence and the type of thermo cycler (Hernendez *et al*., 1999).

Presently, the characterization of plant genetic diversities or relationships is mainly performed based on polymerase chain reaction (PCR) amplification or sequence analysis approaches, including RFLP (restriction fragment length polymorphism), ISSR (inter-simple sequence repeat), RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), and SNP (single nucleotide polymorphisms) (Powell *et al*., 1996; Khlestkina and Salina, 2006). Recently, new approaches such as nrITS (nuclear ribosome internal transcribed spacer) and DNA barcode (such as plastid DNA sequence) (Kress *et al*., 2005) are popularly applied to analyze genetic diversities and relationships. Generally, each approach features its particular sensitivity and applicability. In comparison, RAPD analysis could cover the whole genome information, and is still a convenient, effective and low-cost technology, which has been widely and successfully applied to explore genetic relationships (Dorokhov and Klocke, 1997; Ahlawat *et al*., 2010; Leal *et al*., 2010). Briefly, it could amplify the whole genomic DNA by the PCR technique with a set of 10-nt random primers with each primer having a specific binding site(s) in genomic DNA. Therefore, the information of amplification products by all primers could disclose genetic diversities among samples.

As per literature is concerned, hitherto no work was reported earlier from India on genetic diversity in these four species. Further, before attempting to promote these species as new ornamental, it is essential to establish the relationship among these species. Therefore, more investigations are needed to further explore genetic diversities of *Senecio*.

MATERIALS AND METHODS

The four different species of *Senecio* were collected from Northern parts of Western Ghats. The passport data of the species used for the characterization is given in **Table 1**.

Plant material

Matured leaves were collected from research plot raised for morphological characterization studies. Fresh leaves were quickly brought to the laboratory in butter paper bags where they are swabbed with 76% ethanol to remove traces of dirt and used fresh for DNA extraction.

DNA Isolation

CTAB method (maxi prep) described by Doyle and Doyle (1987) with minor modifications was followed for the isolation of genomic DNA. Freshly harvested young and tender leaf samples (2 g) were ground in liquid Nitrogen using a pre-chilled mortar and pestle. The ground powder was quickly transferred into a clear autoclaved 50 ml centrifuge tube and then 10 ml of pre-warmed (60 ^{o}C) extraction buffer was added and shaken gently to form slurry. The tubes were incubated at 60 ^{o}C in circulating water bath for one hour with intermittent shaking for every 10 minutes with occasional inversion, cold to normal temperature. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed properly by inverting the tubes 20-25 times to form an emulsion and centrifuged at 5000 rpm for 15 minutes at RT to separate the phases. The supernatant was carefully decanted and transferred to a new tube. Again the supernatant was

Table 1. Passport data and morphological traits of the Senecio species used in the study

S. No	*Botanical Name*	*State(Maharashtra)*	*Latitude*	*Longitude*	*Altitude*	*Petal colour*	*FD *(cm)*	*Blooming period*
1.	*Senecio bombayensis*	Durgawadi Ghats	19^{0}12'078"	73^{0}42'473"	826 m	Yellow	2.1-3.6	June – Aug
2.	*Senecio dalzillii*	Durgawadi Ghats	19^{0}11'540"	73^{0}41'417"	1119 m	Yellow	0.8 -1.6	June- Aug
3.	*Senecio belgaumensis*	Panah	16^{0}48'920"	74^{0}06'528"	959 m	Yellow	0.9- 1.4	June- Sep
4.	*Senecio edgeworthii*	Kambakti Ghats	18^{0}24'072"	73^{0}51'412"	887m	Yellow	2.5-3.0	July- Oct

* FD-flower diameter

carefully decanted and transferred to a new tube and was precipitated with two volume of pre-chilled (-20 °C) absolute ethanol and ammonium acetate (final concentration 0.3 M), and gently mixed by inverting up and down (10 minutes) to produce fibrous DNA and incubated at -20 °C for a minimum of one hour.

The samples were centrifuged at speed 5,000 rpm for 5 minutes. Supernatant poured off and the pellet was washed twice and thrice with 70% ethanol. Decanted the supernatant and air dried DNA pellet at RT until the whitish pellet turned to transparent and resuspended in 300 µl of TE Buffer and 6ìl of RNAase (10 µg/µl) was added incubated at 37 °C for two hour (RNAase treatment helped achieving in proper genomic DNA). To this 600 µl of Ice chilled ethanol and 10 ml of 7.5 M Ammonium acetate was added and incubated at -20 °C for one hour to re-precipitate DNA.

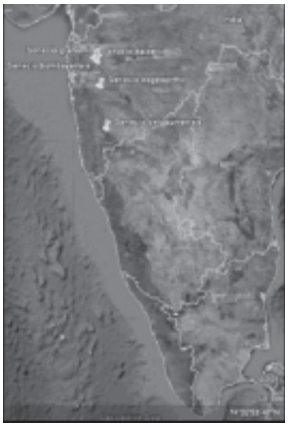

Fig. 1. Location of wild *Senecio* populations samples in northern parts of western Ghats.

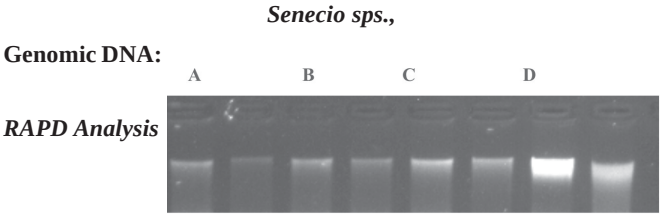

Fig. 2. Genomic DNA of *Senecio* Species

The solution was centrifuged at 10,000 rpm for 15 minutes; All the centrifugation steps were carried out at RT to avoid precipitation with (CTAB, DNA degradation and to obtain good quality of DNA. Centrifuged at 12000 rpm for 20 minutes at 40 °C and decanted the supernatant. The DNA pellet was dried at 370 °C for 15 minute and dissolved in 50 µl of TE buffer. DNA pellet was dried at 37 °C and resuspended in 250 µl of Tris–EDTA (TE) buffer.

Optimization of PCR reactions

For the optimization of RAPD-PCR reaction using DNA extracted from three (S.*bombyence, S.belgaumensis and S. dalzilli*) plant species of *Senecio*, RAPD primers were used for amplification to standardize the PCR conditions. The reactions were carried out in a DNA Thermo cycler. Each 10 µl reaction volume contained about 100 mM Tris-HCl (pH 8.3), 800 mM KCl, 20 mM $MgCl_2$ $6H_2O$, Tritonx -1%, gelatin1%, 1 mM dNTPs, 3 µM primer, 3U/µ Taq Plus DNA polymerase and approximately 40 ng template DNA. The thermo cycler was programmed for an initial denaturation step of 4min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at different annealing temperature for each primer, at 36 °C for 45 Seconds, extension at 72 °C for 1 min and final extension at 72 °C for 10 min and at a temperature of 4 °C at the end. 10 µl of PCR products were electrophoresed on 1.4% (w/v) agarose gels, in 1X TBE Buffer at 80 V for 2 h and stained with ethidium bromide (0.5 µg/ml). Gels with amplification fragments were visualized and photographed in UV light by using Gel Documentation System.

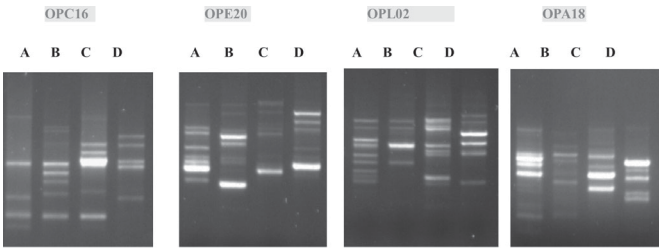

Fig. 3. Gel picture showing the RAPD pattern of Senecio Species from Northern parts of Western Ghats
A- *S. dalzilli, B- S.bombyances, C-S. belgaumensis, D - S. edgeworthii*

Primers are initially screened using pooled DNA (3.5 µl from each) and primers that produced maximum number of scorable bands were selected for RAPD analysis. Amplification profiles of 12 primers **(Table 2)** in 4 species of *Senecio* were scored as presence (1) or absence (0) and the 0, 1 matrix generated is used for statistical analysis.

The amplified products were resolved in 1.2% agarose gel (1´ TBE) followed by EtBr staining and the bands detected were photographed. Amplified products which were reproducible and consistent in performance were chosen for scoring of shared and unshared bands. The presence of a particular band was denoted as 1 and absence as 0. A total of 228 reproducible bands were produced. Out of the amplified products using 12 primers, 68 were found to be polymorphic with an average of 4.6 bands per primer **(Table 2)**. The number of bands produced per primer ranged from 24 (OPC16) to 16 (OPB07), with an average of 19 bands per primer. Multivariate analysis to detect genetic similarity coefficient was performed following Nei and Li's method. Cluster analysis was performed based on the matrix obtained using the computer program PCO3D (version 1.2) and the clusters were represented in the form of a dendrogram **(Fig. 4)**. The present study and similar studies on Lotus, sweet potato, oil palm and potato suggest that RAPD is more appropriate for analysis of genetic variability in closely-related genotypes. The level of genetic variability

detected is moderately high in *S.belgaumensis* with 5.5 polymorphisms revealed per primer. The dendrogram obtained through cluster analysis revealed 2 major clusters.

Table 2. Polymorphism in four Senecio species generated by 12 RAPD primers.

S. No	*Primers*	*Sequence*	*Total Number of bands*	*Number of polymorphic bands*
1.	OPAO4	AATCGGGCTG	18	4
2.	OPA12	TCGGCGATAG	23	12
3.	OPA18	AGGTGACCGT	17	4
4.	OPBO7	GGTGACGCAG	12	0
5.	OPCO8	TGGACCGGTG	21	8
6.	OPC16	CACACTCCAG	24	8
7.	OPE04	GTGACATGCC	16	4
8.	OPE20	AACGGTGACC	23	8
9.	OPGO4	AGCGTGTCTG	17	4
10.	OPL02	TGGGCGTCAA	23	12
11.	OPNo4	GACCGACCCA	16	0
12.	OPO01	GGCACGTAAG	23	4
	Total	228	68	

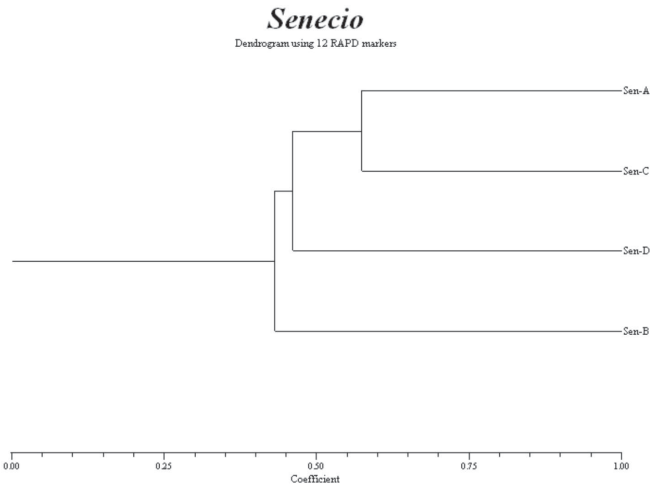

Fig. 4. Dendrogram of four Senecio species collected from northern parts of western Ghats
A- *S. dalzilli, B- S.bombyances, C-S. belgaumensis, D - S. edgeworthii*

RESULTS AND DISCUSSION

RAPD has frequently been used for the detection of genetic variability in plants. The advantages of this method are its rapidity, simplicity and lack of need for any prior genetic information about the plant. RAPD patterns are consistent irrespective of the plant source or age (Welsh and McClelland, 1990; Micheli *et al.*, 1994). This technique has been reported to be useful for the identification and genotyping of ornamental plants (De Benedetti *et al.*, 2001) and other varieties of plant species (Temiesak *et al.*, 1993). The RAPD banding patterns of the four species are illustrated in **Fig. 3**. The 12 primers used for RAPD-PCR were able to amplify the DNA from all the plant species studied. A total of 228 bands were observed for four species using 12 primers. Out of the amplified products using 12 primers, 68 were found to be polymorphic with an average of 19 bands primer **(Table 2)** with 30% of polymorphism. The number of bands produced per primer ranged from 24 (OPC16) to 16 (OPB07).

The minimum genetic similarity **(Table 3)** has been observed (32) between *S. dalzelli and Senecio belgaumensis*, and the maximum genetic similarity (39) was observed between *S. dalzelli and S.edgeworthii and Senecio belgaumensis.* The dendrogram **(Fig. 4)** analysis grouped the 4 plants into two main clusters with 3 species namely *S. dalzelli* (A), *S. belgaumensis* (C) and *S. edgeworthii* (D) in cluster I leaving the *S. bombyances* (B) into cluster II. The cluster I has been further grouped into two sub-clusters, IA with the species *S. dalzilli* (A) and *S.belgaumensis* (C) and IB with *S.edgeworthi* (D).

Table 3. Genetic similarities of four *Senecio* Species of Northern Parts of Western Ghats.

	S. dalzilli	*S. bombyances*	*S. belguamnesis*	*S. edgeworthi*
S. dalzilli	0	38	32	39
S. bombyances		0	34	35
S. belguamnesis			0	39
S. edgeworthi				0

The floral organ for flowering plant, including floral structure (sepals, petals, stamens, and carpels), flower colour and blooming period are considered as the most important traits in horticulture. These traits are strictly spatially and temporally governed and regulated by a series of genes during the course of different developmental phases, which result in the synthesis of flavonoids and carotenoids, pH change of enchylema, development of floral organs, and finally formation of floral structures (Vandenbussche *et al*., 2003; Sablowski, 2010).Three morphological traits, including petal colour, flower diameter and blooming period, were selected for the study. Morphological analysis indicated that all the four species featured similar petal colour. Even though all the species has similar flower colour, the size of the flower and the leaves were very different from each other. RAPD analyses provided insight into genetic diversity, genetic structure and distribution of the four species of *Senecio* under study. It can be observed from the data the even though the natural habitats were different both *Senecio dalzilli* and *S. belgaumensis* were clustered **(Fig. 4 & Table 1)** in one group indicating that both the species share common ancestry.

Morphologically the leaves and plant growth habit were entirely different from each other. *S. dalzilli* had needle type of leaves while *S. belgaumensis* had rhombotic ovate leaves. *S.dalzilli* in its natural habitat was basically prevailing on higher altitudes (1119 m) above mean sea level. Even *S. belgaumensis* were collected from little lower altitudes (959 m). Both the species had very similar type of flowers and the inflorescence looked very much alike. But they differed only in size of the disc diameter. The size of the disc was slightly bigger in case of *S. dalzilli*, while it was small in case of *S. Belgaumensis* **(Fig. 5&6)**. Theoretically, the minimum genetic similarity (32) was observed between these two species. *S. edgeworthii* clustered along with *S. dalzilli* and *S. belgaumensis*. But morphologically *S. edgeworthii* had very distinctive leaf colour and flower size and number of ray florets. Blooming period with respect this species falls into late group compared to rest of the three species, while rest three groups fall under early blooming period. Maximum genetic similarity (39) was observed between *S. dalzilli, S. belgaumensis and S. edgeworthii*. The clustering pattern of *S. bombyances* indicates its genetic variability which might be due to different ancestry from the other 3 species. *S. bombyances* had a very big size of flowers and the leaves were very different in shape **(Fig. 6 & 7)**. Therefore, RAPD, analysis was sensitive to distinguish genetic diversities among *Senecio* species.

Different molecular marker approaches or techniques have their particular sensitivity and applicability. In comparison, the RFLP approach covers a small volume of information, ISSR markers have limited binding sites to genomic DNA, and AFLP markers feature a complicated operation process due to relatively high stability. For SNP analysis, it is costly and labour-intensive, though the approach could disclose

Fig. 5. *Senecio dalzilli (* Hook)

Fig. 6. *Senecio belgaumensis (Wight)*

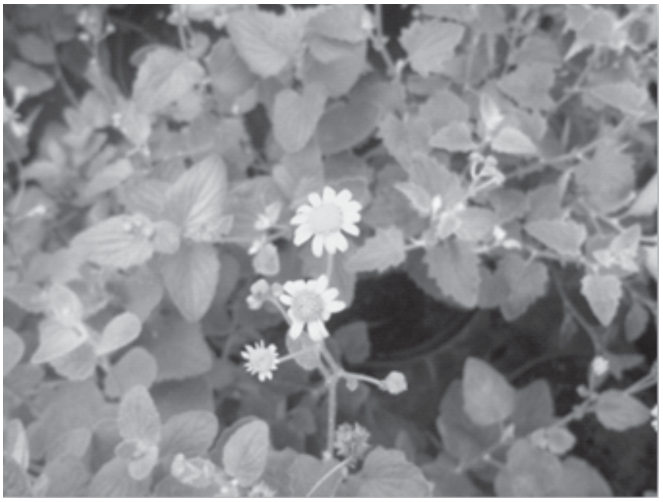

Fig. 7. *Senecio edgeworthii.*(Hook)

Fig. 8. *Senecio bombayences* (Balakr)

relatively true genetic diversities. Recently, new approaches such as nrITS and plastid DNA sequence analysis have been applied to explore genetic diversities, but both approaches also disclose a small volume of local information of the genome. For RAPD markers, there are some doubts because of their relatively low reproduction (or stability). However, this problem of reproduction (or stability) could be easily solved by using high-quality genomic DNA, optimized reaction system and program in a standard operation. In the present study, clear, stable and reproducible amplification bands were obtained with an optimized system and procedure (Wang *et al*., 2010). Therefore, RAPD markers still provide a rapid and useful technique to investigate genetic diversities at the whole genome level (Ahlawat *et al*., 2010; Ahmad *et al*., 2010; Leal *et al*., 2010).

This study used RAPD technique to produce basic genetic information about natural populations of *Senecio* of Northern parts of Western Ghats. There is still no set of genetic diversity data obtained by one method and covering all of Indian origin *Senecio* diversity. To obtain detailed genetic information that will facilitate conservation and management of *Senecio* species, future studies should be wide-ranging and should apply fine scale analysis using molecular markers. RAPD markers may offer an additional source of data for assessing genetic diversity within and relationships among closely related species. In this study, based on a comprehensive analysis of morphological traits (petal colour, flower diameter, blooming period) and genetic diversities, it was found that the *S. dalzelli, S. belgaumensis, S. bombyances* seems to be most suitable for further breeding programmes.

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Influence of low temperature storage techniques on post-storage quality of rose cut flowers *var*. Passion

Alka Singh*, R. J. Makwana and B. D. Mangave

Department of Floriculture and Landscape Architecture, Navsari Agricultural University, Navsari – 396450 (Gujarat).
**Corresponding author: dralkasinghdhaka@gmail.com*

ABSTRACT

Influence of different storage techniques *viz*., seal packaging with polypropylene (PP 24µ), butter paper (52µ), holding in 200 $mgL^{-1}Al_2(SO_4)_3$ vase solution, holding in vase (distilled water) and without any packaging and without holding in vase water (as controls) on post storage quality of rose cut flowers var. Passion was investigated at low temperature (2 °C) for 10 days. The polypropylene packaged low temperature stored cut roses showed promising result with best postharvest flower quality at the end of 10 days storage period as compared to other treatments. The PP packaged low temperature stored rose cut flowers showed, negligible physiological loss in weight, retained higher petal sugar levels, showed higher water uptake, absence of bent neck, retained higher anthocyanin pigment contents and petal size when held in vase (distilled water) after 10 days of low temperature storage compared to all treatments. Cut flowers held in vase solution during low temperature storage showed advance opening of flowers with decrease in vase life upon removal from low temperature storage. PP packaged low temperature stored cut roses showed higher percent absolute integrity (PAI) of cell membrane in petal tissue that delayed senescence and enhanced vase life as compared to all other treatments including controls.

Keywords: Rose, Polypropylene, low temperature storage, anthocyanins, bent neck, PAI, vase life

INTRODUCTION

Roses are the most popular of all cut flowers in the world. The demand of cut roses often reaches its peak followed by high prices during festival times while at times faces the problem of price crash during market gluts. Effective storage technique and long distance transportation can facilitate better market strategy for roses. However, long distance transportation of roses via sea shipment is further restricted due its limited vase life, deterioration in flower quality (Mor *et al*., 1989, van Doorn and d' Hont, 1994) and chilling injury (Pompodakis *et al*., 2010) at low temperature storage. Further, low temperature storage has also been known to promote ethylene production that further triggers early senescence in rose (Faragher *et al*., 1986, Devechhi *et al*., 2003). Seal packaging of cut flowers with polyfilms at low temperature is known to create modified atmospheric conditions (Farber *et al*., 2003) that has been found to play an important role in retaining flower quality, improving opening ability, reducing water loss during post storage phase in stored flowers like gladiolus (Grover *et al*., 2005, Singh *et al*., 2007), *Solidago canadensis* (Zeltzer *et al*., 2001) and in Lisianthus (Akbudak *et al*., 2005).

Research on storage aspects in roses needs to be further studied. Hence, this experiment was planned to evaluate proper low temperature storage technique for rose cut flower that would aid in the development of market strategy and accessibility at International market. Different storage techniques *viz*., wet storage in 200 $mgL^{-1}Al_2(SO_4)_3$ solution and water (tap or DW) and dry storage consisting of two packaging films, *viz*., polypropylene (PP; 24 µ) and butter paper (52 µ) and without any packaging (as controls) were investigated for cut roses var. Passion.

MATERIALS AND METHODS

Plant material and location

Fresh rose cut flowers *var*. Passion were obtained from greenhouse complex, Navsari Agricultural University, Navsari and were brought to the Floriculture laboratory, College of Horticulture and Forestry, NAU Navsari at an ambient temperature (18-21 °C). The experiment was conducted in completely randomized block design. There were five treatments and each treatment was repeated four times. Cut roses at uniform bud size, fresh weight (10±2 gm) and stem length (50±5 cm) were selected and divided into five groups each having one hundred twenty flowers (30 in each replicate) and exposed to different treatments.

Packaging and low temperature storage

Each group of one hundred twenty flowers was again

divided into twelve bunches having ten flowers each. Each group having twelve bunches of flowers (3 bunches in each replication) were given different treatments like seal packaging with polypropylene 24 microns (PP) (T_1), seal packaging with butter paper 52 microns (T_2), holding in 200 mgL^{-1} $Al_2(SO_4)_3$ solution (T_3), holding in distilled water (T_4) and remaining twelve bunches were kept without any packaging and holding solution (T_5) in CFB box as control and all the bunches were stored at 2 °C for 10 days. After 10 days of low temperature storage, the flowers were held in distilled vase water at ambient conditions in the laboratory for recording of different observations.

Observations recorded

Observations on vase life and different postharvest parameters at different intervals in vase were recorded during vase life. Physiological loss in weight was estimated just after removal of rose flowers from cold storage in per cent on the basis of initial fresh weight. Water uptake was recorded on 2nd and 4th day of vase life. Total soluble sugars, petal length, petal width and pigment content were recorded just after storage. Per cent bud opening and bent neck was recorded just after storage, on 2nd and 4th day of vase life. Absolute integrity of cell membrane was estimated in per cent just after storage, on 2nd and 4th day of vase life after storage on the basis of electrolyte leakage.

Physiological loss in weight and Water uptake

Physiological loss in weight (%) of flowers was calculated by given formula

$$PLW = \frac{(Initial\ weight — Final\ weight)}{Initial\ weight} \times 100$$

The water uptake by the cut rose flowers after storage was recorded at regular intervals and volume of water used was depicted in ml.

Total Soluble Sugar (TSS) (mg/g fresh weight)

One ml of water was used to measure the TSS of the petals from the solution prepared for electrolyte leakage as per the method given by Franscistt (1971). In 1 ml solution, 4 ml of 0.2% anthrone reagent (200 mg in 100 ml H_2SO_4) was added in each test tube and was placed in ice cold water. Reagent blank was prepared by adding 1 ml of distilled water and 4 ml of 0.2% anthrone reagent. The intensity of colour was measured at 600 nm on spectrophotometer. A standard curve was prepared using 10 mg glucose per 100 ml distilled water.

Total soluble sugar (mg/g) = sample O.D. x Standard O.D. x dilution factor

Percent Bud Opening

Percent bud opening was measured on the basis of flower bud diameter and converted it into percentage basis by using following conversion table

Flower diameter (cm)	% opening
>5	100
5-4.5	80
4.5-4	70
4-3.5	60
3.5-3	50
3-2.5	30
2.5-2	20
2-1.5	0

Petal size

Petal size was measured in terms of length and width from the centre of the outer most petal in cm using vernier caliper.

Anthocyanin Content

Anthocyanins were extracted from the petals and determined following the method described by Lees and Francis (1972). A sample of 2 gm weighed rose petals was taken from the centre of the bud and was macerated nicely with the help of a mortal and pastel. It was mixed with 20ml of mixture 95% ethanol: 1.5 N HCL (In the proportion of 85:15). This mixture was transformed in to another beaker, covered with para-film and stored overnight at 4 °C. Next day; the mixture was filtered through No.1 Whatman filter paper in a funnel and the filtrate was collected in a flask. After the filtration was over, the macerate (left in the filter paper) was again mixed with 10 ml of extracting solvent and filtered through another No.1 Whatman filter paper into the flask containing earlier filtrate. The final volume was made to 30ml by the addition of extraction solvent. From that solution, 10ml aliquot was taken into another beaker and volume was made 20ml by the addition of extracting solvent. This solution, was then stored in dark for two hours at room temperature and the spectrophotometer reading was recorded at 535nm wavelength and the anthocyanin pigment was estimated according the formula:

Anthocyanin content (mg/g) = O.D535 X Dilution factor X 10/AvgE1%535

= (O.D535 X Dilution factor)/98.2.,

Dilution factor = (original extract X dilution amount)/ extract taken for dilution

Bent neck (angle degree)

In rose cut flowers, longitudinal axis of the flower was considered as 0^o (degree) and angle of bent was measured (in degree) with the help of protector just after storage, 2nd and 4th day of vase life (after 10 days of storage).

Absolute integrity of cell membrane

Absolute integrity of cell membrane was calculated on the basis of electrolyte leakage of petals using conductivity meter according to the method described by Azevedo *et al*. (2008). The percentage of absolute integrity (PAI) of cell membranes was determined from one flower in each replicate. Five discs (10 mm diameter) were sampled from the tissue of the second whorl of open petals. The discs were kept in test tubes immersed in 30 ml of distilled water for 24 h prior to the first measurement of free conductivity. The second measurement (total conductivity) was performed 1h later in test tubes that remained in a water bath at 100 ^oC. PAI of the cell membrane was calculated using below given formula:

$$PAI = \left(1 - \frac{FC}{TC}\right) \times 100$$

where, PAI is the percentage of absolute integrity of cell membranes (%),

FC the free conductivity (dS m^{-1}) and

TC is the total conductivity (dS m^{-1}).

Vase life

Vase life was measured in days after removal of rose cut flowers from cold storage and held in vase water until the wilting and fading of outer 1-2 petals.

Statistical analysis

The recorded data were statistically analyzed (ANOVA analysis) using the software WASP, (developed at ICAR Research Complex for Goa, India). Source of variation were storage treatments. Mean comparisons were performed using LSD test to determine whether the difference between the variables were significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Physiological weight loss, Total soluble sugars and water uptake

Seal packaging of rose cut flowers were highly effective in retaining postharvest physiology that contributed in improved post-storage flower quality and vase life of rose cut flowers after 10 days of low temperature storage. Storage techniques significantly influenced physiological loss in weight, total soluble sugar just after storage and water uptake on alternate days when the cut flowers were held in water upon removal from low temperature storage **(Table 1)**. PP packaged low temperature stored rose cut flowers showed negligible physiological loss in weight and higher retained petal sugar status as compared to other dry storage techniques. Cut roses sealed packed in poly-propylene (T_1) recorded minimum physiological loss in weight (1.55%) and maximum water uptake on 2nd and 4th day after storage (60.14 and 50.62 ml respectively) which was followed by flowers held in $Al_2(SO_4)_3$ 200 mgL^{-1} (T_3). Further, polypropylene seal packed flowers also recorded maximum total soluble sugars in petal (5.53 mg/g fresh weight), followed by flowers held in a vase solution containing $Al_2(SO_4)_3$ 200 mgL^{-1} (T_3) (4.23 mg/g FW) just after storage. Maximum physiological loss in weight (34.70%) and lower total soluble sugar in petal (3.04 mg/g FW) just after storage was recorded in control (T_5), followed by butter paper packaging (T_2).

Table 1. Effect of different storage techniques on Physiological Loss in Weight (PLW), water uptake and petal sugar content of rose cut flowers var. Passion.

Treatment	*PLW (%)*	*Water uptake (ml)*		*Total soluble sugar (mg/g FW)*
		2nd day	*4th day*	
T_1- Polypropylene	1.38	60.14	50.62	5.79
T_2- Butter paper	23.46	12.22	-	3.23
T_3- 200 mg/l $Al_2(SO_4)_3$	3.92	46.95	42.94	4.23
T_4- Water	4.91	35.67	-	3.82
T_5- Without any packaging or holding solution (Control)	34.70	8.42	-	3.04
LSD at 5%	0.23	2.65	1.23	0.26

Seal packaging of fresh produce in poly films is known to create modified internal gaseous components passively (Farber *et al*., 2003), that helps in minimizing metabolic activities during storage and retains fresh produce in normal condition (Zeltzer *et al*., 2001). PP packaging might have thus, contributed to minimal cell damage during storage and retaining of normal cell condition after storage as indicated by minimal physiological loss in weight, retained petal sugar status and high membrane integrity of petal tissue **(Table 3)**. These conditions further resulted into higher water uptake in PP packaged cut roses when held in vase water after storage as compared to other treatments. The increase in the fresh weight in wet stored [$Al_2(SO_4)_3$ and water both] rose cut flowers was a result of continuous supply of water during the storage period which contributed in maintaining water balance and higher fresh weight as recorded just after storage. Low water uptake in wet stored and dry stored (butter paper and controls) cut roses after

storage was due to gradual advancement in senescence stage as evident from short vase life **(Table 3)**. Direct relationship of water uptake with vase life has been well known (Halevy, 1976). Further, flowers show restricted water uptake after storage due to damaged xylem structure on account of desiccation during low temperature storage (van Doorn and D'Hont, 1994 and Faragher *et al*., 1984). Packaging with poly films have been earlier known to enhance water uptake after cold storage in gladiolus cut spikes (Singh *et al*., 2007 and Grover *et al*., 2005), gerbera (Patel and Singh, 2009) and solidago (Zeltzer *et al*., 2001).

Bud opening percentage and petal size

All the storage treatments significantly influenced bud opening and petal size in rose cut flower var. Passion. Just after storage, minimum bud opening (retained bud stage) was recorded in polypropylene packaged flowers while wet stored flowers showed higher opening (74-90 %) as shown in **Table 2 & Fig. 1**. However, the PP packaged flowers opened rapidly and recorded maximum opening (100%) on 4th day when held in vase upon removal from storage and packaging as compared to all treatments and control. Petal size was recorded in terms of length and width of petal **(Table 2)**. The maximum petal length (3.97 cm) and width (3.6cm) was detected in rose cut flowers seal packaged in polypropylene (PP) (T_1) which was followed by cut flowers wet stored in $Al_2(SO_4)_3$ (T_3). Minimum petal length (2.57 cm) and width (2.43 cm) was recorded in flowers kept without packaging and holding solution (T_5) followed by Butter paper packaging (T_2).

Enhanced bud opening along with improved petal size of cut roses packaged with PP during low temperature (2 °C) was due to availability of water through improved water

Table 2. Effect of different storage techniques on bud opening, petal length, petal width and anthocyanins of rose cut flowers var. Passion.

Treatment	*Bud opening (%)*			*Petal length (cm)*	*Petal width (cm)*	*Anthocyanins (mg/g)*
	Just after storage (JAS)	*2nd day*	*4th day*			
T_1- Polypropylene	0.82	80.23	100.00	3.97	3.6	8.12
T_2- Butter paper	27.94	36.34	-	2.77	2.57	5.94
T_3- Al2(SO4)3 200 mg/l	90.13	92.34	95.63	3.9	3.03	7.61
T_4- Water	74.34	85.00	-	3.53	2.67	6.17
T_5- Without any packaging	23.42	36.04				
or holding solution (Control)	-	2.57	2.43	3.99		
LSD at 5%	2.71	3.30	2.94	0.38	0.32	0.38

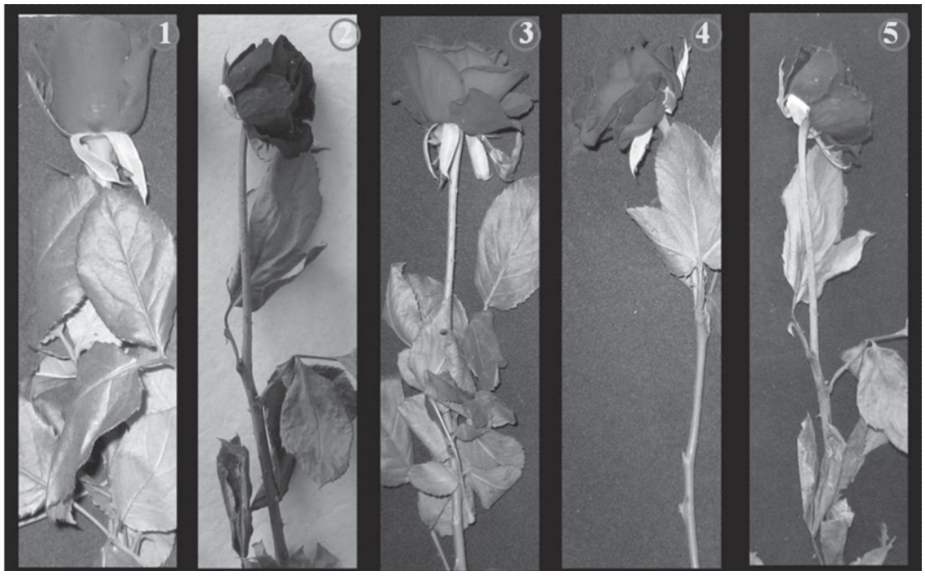

Fig. 1. Influence of different storage techniques on postharvest quality of rose cut flower after 10 days of low temperature storage, where (1) PP packaged low temperature stored (2 °C) roses, (2) Butter paper packaged low temperature stored (2 °C) roses, (3) Wet storage in Aluminum sulphate solution, (4) Wet storage in water and (5) Control (without any packaging and holding). Photograph was taken just after 10 days of storage.

uptake when held in vase water after storage. Further, the retention of high petal sugar level (higher TSS) along with improved membrane integrity of petal cells **(Table 3)** contributed to higher bud opening and petal size. Water balance (Halevy and Mayak, 1981) along with intact cell membrane of petal tissue (Torre *et al*., 1999) was suggested to improve bud opening in cut flowers. Bud opening of cut flower has also known to be associated with petal sugar status (van der Meular *et al*., 2001, Singh *et al*., 2009). Further, the role of sugars in floret expansion by facilitating respiration necessary for cell division and cell enlargement is well known (Ho and Nichols, 1977). Butter paper packaged and dry stored (without any packaging) cut roses showed significant decrease in petal size and bud opening due to desiccation and failure of buds to open due to water and sugar stress.

synthesis in plant cells (Leyva *et al*., 1995). However, influence of storage conditions including low oxygen (Markakis, 1982, Attoe and von Elbe, 1981) and high relative humidity (Somboonkaew and Terry, 2008) have been found on anthocyanin retention in different plant products. Thus, modified atmospheric condition with PP polyfilm packaging retained anthocyanin pigments in the low temperature stored rose petals as earlier observed in gladiolus (Singh *et al*., 2009).

PAI of petal tissue, bent neck and vase life

Significant effect of storage techniques on bent neck, absolute integrity of cell membrane and vase life was recorded in rose cut flower *var*. passion **(Table 3)**. Minimum bent neck (0.830, 7.670 and 28.57°) just after storage, 2nd and 4th day after storage and maximum percent absolute

Table 3. Effect of different storage techniques on bent neck, absolute integrity of cell membrane and vase life of rose cut flowers var. Passion

Treatment	*Bent Neck (°)*			*Absolute integrity of cell membrane (%)*			*Vase life*
	JAS	*2nd day*	*4th day*	*JAS*	*2nd day*	*4th day*	*(days)*
T_1- Polypropylene	0.83	7.67	28.57	89.53	62.27	38.63	5.07
T_2- Butter paper	23.47	77.43	-	36.27	27.37	-	1.30
T_3- Al2(SO4)3 200 mg/l	6.23	22.93	65	75.87	51.63	26.67	3.23
T_4- Water	9.07	28.43	-	68.03	42.13	-	2.33
T_5- Without any packaging or holding solution (Control)	38.57	83.63	-	32.63	26.07	-	1.03
LSD at 5%	1.26	1.86	1.65	1.82	2.05	1.44	0.25

Anthocyanin pigments in petals

All the treatments significantly influenced anthocyanin pigment content after storage. PP packaged low temperature stored rose cut flowers showed higher retained anthocyanin content as compared to flowers stored with other techniques **(Table 2)**. The maximum pigment (anthocyanin 8.12 mg/g FW) in rose petals was recorded in polypropylene seal packaged (T_1). While, minimum anthocyanin pigment (3.99 mg/g FW) was recorded in petals of flowers stored without any packaging followed by butter paper packaging.

Sugars serve protective role for vacuolar structure (Yu, 1999), and the site for location of anthocyanins and stimulating anthocyanin synthesis is plant cells (Weiss, 2000). Sugars have also been found to protect anthocyanins from degradation during low temperature storage and also prevention of browning (Wrolstad *et al*., 1990). Thus, retention of sugars in the petal cells of PP packaged cut roses contributed to retained anthocyanin pigments in the petals of rose cut flowers. Correlation of petal sugar levels and anthocyanins has also been known and indicated earlier in many flowers (Grover *et al*., 2005, Singh *et al*., 2009).

Further, low temperature is known to trigger anthocyanin

integrity of cell membrane in petal tissue (89.53, 62.27 and 38.63% just after storage, 2nd and 4th day after storage) was recorded in cut flowers seal packaged in polypropylene (T_1), which was followed by flowers wet stored in 200 mg/l $Al_2(SO_4)_3$ (T_3). Flowers stored without packaging recorded maximum bent neck (38.57° and 83.63° just after storage and on 2nd day after storage) and minimum absolute integrity of cell membrane (32.63° and 26.07% just after storage and on 2nd day after storage). The maximum vase life (5.07days) was recorded in cut flowers seal packaged with polypropylene followed by flowers wet stored in 200 mgL^{-1} $Al_2(SO_4)_3$ (T_3, 3.23 days) while minimum vase life (1.03 days) recorded in flowers stored without packaging (T_5).

High percent absolute integrity of cell membrane of petal tissue and absence of bent neck in PP packaged low temperature stored cut flowers was the result of high water uptake and high petal sugar status. The adequate levels of water balance and carbohydrate content in the petal cells of the polypropylene packaged flowers retained the bio-membrane fluidity and permeability and thus contributed in high membrane stability of the rose petal tissue as also reported by Singh *et al*. (2007) in gladiolus. A strong relationship of water imbalance with bent neck in roses

has been well known (Zieslin *et al*., 1978, Izumi *et al*., 2000). One of the positive effects of modified atmosphere conditions during low temperature storage is the reduction of water loss from the produce (Saltveit, 1997). This was also observed in packaged low temperature stored rose cut flowers (as indicated by fresh weight retention during storage) that resulted into alleviation of any bent neck symptoms. Further, increase in electrolyte leakage for petal tissue along with water stress has also been linked to bent neck in roses (Mayak and Halevy, 1974, Izumi *et al*., 1997) and early senescence in gladiolus (Singh *et al*., 2008) and gerbera (Patel and Singh, 2009).

Thus, the retention of normal petal cell conditions as indicated by negligible physiological weight loss, higher petal sugar status, better water balance with high water uptake and improved PAI of petal cell tissue alleviated bent neck conditions that delayed petal senescence and improved vase life of PP packaged low temperature stored cut roses.

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Growth promotion and management of *Alternaria* leaf spot in chilli by *Trichoderma harzianum*

Alok K. Srivastava*, Ram Nageena Singh, Sudheer Kumar, Prem Lal Kashyap and Dilip K. Arora

National Bureau of Agriculturally Important Microorganisms, Kusmaur, Mau, 275101, India
**Correspondence: aloksrivastva@gmail.com*

ABSTRACT

Chilli (*Capsicum annum*) leaf spot caused by *Alternaria solani* is the most important, devastating and widespread disease in India. *In vitro* and greenhouse screening of *Trichoderma harzianum* isolates (T. har 25-92 and T. har 29-92) was conducted to investigate the plant growth promoting activities and inhibition against *A. solani* in chilli. Dual cultures of *T. harzianum* isolates and *A. solani*, with and without contact, were used as a mean to study the mycoparasitic response in T. har 25-92 and T. har 29-92. Both the isolates produced significant amount of chitinase and β-1, 3 glucanase in response to pathogen contact. Greenhouse studies indicated that T. har 25-92 and T. har 29-92 exert strong antagonistic activity against *A. solani*. Additionally, a significant increase in per cent seed germination and growth of chilli plants in terms of plant height, fresh weight, dry weight, and chlorophyll content was recorded. The induction in the level of total phenolic content and peroxidase activity by 1.5 to 3.0 fold in T. har 25-92 and T. har 29-92 treated plants indicates that these enzymes may have a role in the defense response against *A. solani*. Overall, the results suggest that these strains may be exploited for management of leaf spot and growth promotion in chilli.

Keywords: Chilli, chitinase, leaf spot, PGPR, *Trichoderma*

INTRODUCTION

Leaf spot disease caused by *Alternaria solani* is one of the major constraint for successful cultivation of chilli in India. The pathogen has been reported to infect seed, foliage and fruit (Kumar *et al.*, 2013). Post-harvest decay of fruits and seeds has also been recorded due to this pathogen (Singh and Rai, 2003). However existing commercial cultivars of chilli do not provide sufficient disease resistance. Effective fungicides are available to manage the pathogen (Kumar *et al.*, 2013), but the increased environmental concern over their use has necessitated a large upsurge of biological disease control. The emergence of fungicide resistance among the pathogens, ground water and food pollution and development of oncogenic risks has further encouraged the exploitation of potential antagonistic microûora in disease management. Among various antagonists used for the management of plant diseases, *Trichoderma* play a vital role (Solanki *et al.*, 2011).

Trichoderma spp. have revolutionized the field of biological control of plant pathogens (Radjacommare *et al.*, 2010). Intensive research on biocontrol with *T. harzianum* has been carried out to manage bacterial and fungal diseases of greenhouse crops (Elad and Shtienberg, 1995; Srivastava *et al.*, 1996; Freeman *et al.*, 2004; Begum *et al.*, 2010). The first biocontrol agent (BCA) commercialized, registered and used in greenhouse crops was isolate T-39 of *T. harzianum* (TRICHODEX), which effectively controlled *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Cladosporium fulvum* diseases in greenhouse-grown tomato and cucumber (Elad, 2000). The application of *T. harzimum* Th-1 as biocontrol agent, not only resulted in reduced disease severity of *A. alternata* causing black fruit spot on persimmon fruits but also enhanced plant growth (Batta, 2004). The efficacy of *T. harzianum* Th-3 has been most extensively studied on horticulture crops such as cauliflower, cabbage, onion, garlic, chilli, rose and gladiolus against a large number of foliar and soil borne fungi such as *Pythium aphanidermatum, Sclerotinia* spp., *Rhizoctonia solani*, *Colletotrichum* spp., *Alternaria porri* and various other *Alternaria* spp. under *in vitro* and field conditions (Sharma and Sain, 2005; Sharma *et al.*, 2005; Prakasam and Sharma, 2012).

Parasitism of pathogenic fungi, facilitated by the production of hydrolytic enzymes, is involved in biological control of fungal diseases (Solanki *et al.*, 2011; Kumar *et al.*, 2012). *Trichoderma* can improve plant growth and development through mycoparasitism, antibiosis, competition, induced plant defense and act as avirulent symbionts (De Souza *et al.*, 2008; Harman *et al.*, 2004). Moreover, application of *Trichoderma* spp. restricts the growth of pathogens by secreting various extracellular enzymes such as chitinases (Almeida *et al*., 2007), β-glucanases (Haran *et al.*, 1995) and proteinases (Geremia *et al*., 1993). In spite of enormous

scientific research on biological control of plant pathogens with *Trichoderma* spp., the most effective species and their mode of action against *A. solani* has yet to be deciphered. Hence, in the present study, potential of *T. harzianum* 25-92 and *T. harzianum* 29-92 was tested for the biocontrol of *A. solani* and effect of pathogen and antagonist on seed germination, growth and root colonization. Further, the hydrolytic enzymes of test isolates were characterized to study the mechanism of interaction and their impact on chilli vigour.

MATERIALS AND METHODS

Microorganism used and maintenance

Trichoderma harzianum 25-92 (T. har 25-92) and *T. harzianum* 29-92 (T. har 29-92) (Jyotsana *et al.,* 2008) were used as biocontrol agent against *Alternaria solani*. Virulent isolate of *A. solani* was isolated from the infected chilli plants, purified and tested for pathogenecity under glass house conditions. Pathogen and biocontrol agents were cultivated on potato dextrose agar (PDA) at 25 °C for 5-7 days and stored at 4 °C for further use.

Characterisation of *T. harzianum* isolates for enzyme production

The isolates of *T. harzianum* were characterized for induction of hydrolytic enzymes (chitinase and β-1, 3-glucanase) in dual culture confrontation assay with *A. solani*. Chitinases and β-1, 3-glucanases activity of *T. harzianum* isolates were determined up to 4 days after initial contact of *A. solani* with *T. harzianum* isolates in PDA plates (Yedidia *et al.,* 2001). Assay of chitinases was based on determination of *p*-nitriphenyl cleaved from a chitin analogous substrate *p*-nitrophenyl-β-D-N-N'-diacetylchitobiose (PNP) (Roberts and Selitrennikoff 1988). β-1,3-glucanase activity was estimated using the procedure of Koga *et al*. (1988).

Mass multiplication of pathogenic fungi and biocontrol agent

Mycelial discs (5 mm diameter) of *A. solani* were obtained from 4-5 days old culture and separately transferred to 50 ml PDA in 250 ml conical flask and incubated at 28 °C. After incubation, 30 ml of sterile distilled water was added to each culture and the flasks were shaken at 50 rpm for 30 min in an orbital shaker. The content of each conical flask was filtered through sterile muslin cloth. The culture filtrate, containing the spores, was collected, and a concentration of 5×10^8 spores/ml was obtained by dilution with sterilized distilled water.

For the propagation of biocontrol agents, different isolates of *T. harzianum* were multiplied in the broth containing molasses (30 ml), KH_2PO_4 (2 g), $MgSO_4$ (0.2 g), sucrose (10 g) in 1L distilled water (pH: 5.8). After incubation (10 days), conidia of the *T. harzianum* were separated from mycelia by filtering though sterile glass wool and quantified by using a haemocytometer and adjusted the conidial density to 5×10^8 spores ml^{-1} to be used for the seedling treatments.

Seed germination assay

Seeds of chilli (*Capsicum annum* L.) were surface sterilized by treating with 70% ethanol for 2 min followed by 2% NaOCl for 2 min and washing thrice with sterile water, and soaked in sterilized distilled water for 12h. The water soaked seeds were transferred to the petri dishes (10 seed $plate^{-1}$) containing sterilized sand (10 mm) mixed with conidial suspension (10^6 spores ml^{-1}) of T. har 25-92 and T. har 29-92. Petri dishes were incubated at 28±2 °C in dark. Percent germination was recorded after 6th day of incubation upto15 days.

Greenhouse experiment for growth promotion and biocontrol

Germinating chilli seeds were transferred to plastic pots (2 seedlings pot^{-1}) half filled (400g pot^{-1}) with sterilized loamy soil (organic matter 5%) mixed with acid washed sand (1:1; w:w). Before transplantation, spores of T. har 25-92 and T. har 29-92 were mixed directly to the soil (~10^8 spores g^{-1} soil) before filling in pots. The pots were watered and upper surface of sand was covered by 3 cm thick and dense layer of sterilized perlite and kept in green house (28-30 °C day and 24 °C night temperature). The plants were irrigated twice daily with tap water. Experiment was arranged in a completely randomized design (CRD) in five replicates. Observations involved various plant growth parameters namely root and shoot length, root and shoot fresh weight, root and shoot dry weight, and chlorophyll content for the different treatments. Chlorophyll content in leaves was measured as spad units determined by a SPAD-502 chlorophyll meter (Minolta, Japan).

Disease assessment

The biocontrol ability of *T. harzianum* was evaluated in greenhouse experiments. Conidial suspension of *A. solani* (5×10^6 conidia ml^{-1}) was sprayed on the leaves of one month old chilli plants. The observations for disease incidence were recorded at 7, 14, 21 and 28 days post inoculation. Disease was graded using the following scale: 1= healthy plants, no symptom; 2= browning pinhead like spots on leaves; 3= dark brown to black spots with concentric ring and halo; 4= burnt leave with heavy infection. Lesions on the entire foliage and the disease severity index were calculated as described by Bull *et al.*

(1991). The root colonizing population of T. har 25-92 and T. har 29-92 was determined by dilution plating of the soil samples collected at weekly interval up to 28 days post inoculation of biocontrol agent.

Biochemical basis of plant defense

The effect of T. har 25-92 and T. har 29-92 on the induction of plant defense system was evaluated by estimation of peroxidase and total phenols. The leaves of chilli plants from various treatments were collected at 0, 1, 2, 3, 4 and 5 days post inoculation (dpi).

For the estimation of peroxidase activity, chilli leaves (1g) were homogenized in 2ml of 0.1 M phosphate buffer (pH 7.0) at 4 °C. The homogenate was centrifuged at 16000 rpm (4 °C for 15 min) and supernatant used as the enzyme source. The reaction mixture contained 1.5 ml of 0.05 M pyrogallol (Hi-media, India), 0.5 ml of enzyme extract and 0.5ml of 1% H_2O_2 (Hi-media, India). The reaction mixture was incubated at room temperature (28±2 °C). The changes in absorbance at 420 nm were recorded at 30s intervals for 3 min. The enzyme activity is expressed as the changes in absorbance min^{-1} mg^{-1} protein (Ramanathan and Vidhyasekaran, 1997).

For the estimation of total phenol, plant leaves (1 g) were homogenized in 10 ml of 80% methanol (v:v) and agitated for 15 min at 70 °C. One ml of methanolic extract was added to 5 ml distilled water and 250 µl Folin-Ciocalteau reagent (1N) and kept at 25 °C. The absorbance of the developed blue colour was measured at 725 nm with catechol as the standard. The amount of phenolics is expressed as catechol μg^{-1} g^{-1} fresh wt. (Zieslin and Ben-Zaken, 1993).

Statistical Analysis

All experiments and treatments were arranged in complete randomised block design (CRD) with five replicates for each treatment. The data were statistically evaluated using ANOVA for *in vitro* quantification of hydrolytic enzymes of *T. harzianum* isolates and means were compared by using Tukey–Kramer post hoc test ($p \leq 0.05$), using SPSS 16.0 software (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Characterisation of hydrolytic enzymes of *T. harzianum*

A significant accumulation of chitinases and β-1, 3-glucanase enzymes was recorded in T. har 25-92 and T. har 29-92 isolates **(Fig. 1&2)** during the confrontation with *A. solani*. In general, the level of chitinase increased by 1.5-3 fold up to four days after initial contact; thereafter, a slight decrease in the chitinases activity was observed **(Fig. 1).** Similar trend was observed in case of β-1, 3-glucanase activity **(Fig. 2)**. At four days post contact with *A. solani*, T. har 25-92 and T. har 29-92 produced 2.9 and 3.1 U/mg

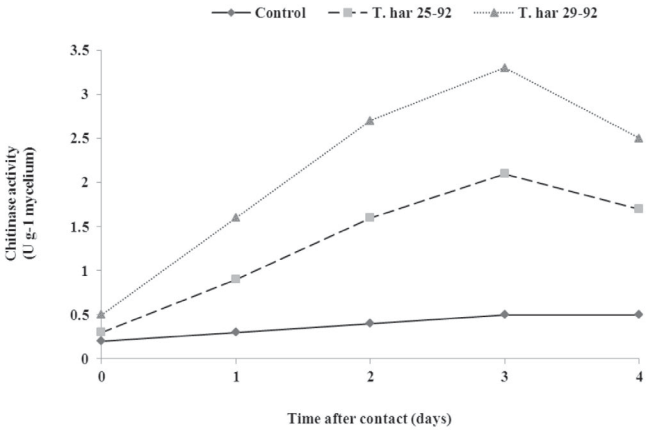

Fig. 1. Chitinase activity of T. har 25-92 and T. har 29-92 at 0, 1, 2, 3 and 4 days post contact with *A. solani*.

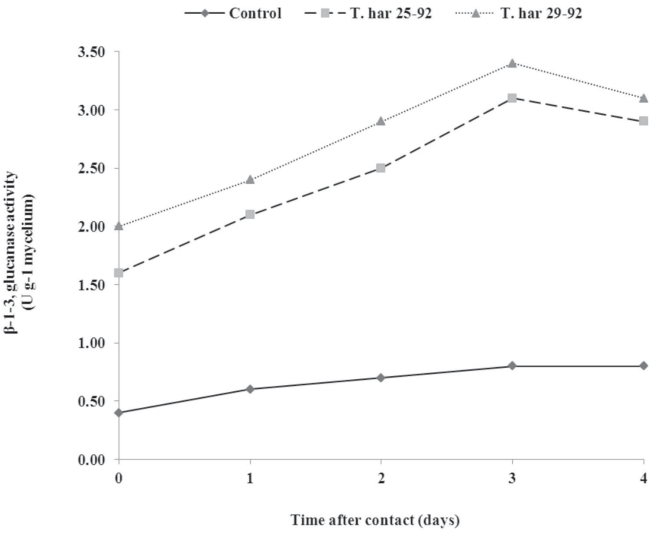

Fig. 2. β-1, 3 glucanase activity of T. har 25-92 and T. har 29-92 at 0, 1, 2, 3 and 4 days post contact with *A. solani*.

β-1, 3-glucanase, respectively.

Plant growth promotion

Seed germination was significantly enhanced by the treatments **(Fig. 3)**. The highest percentage of seed germination (90%) was recorded when the seeds were treated with T. har 29-92 followed by T. har 25-92. Whereas, poor seed germination (70%) was recorded in control **(Fig. 3)**. The results pertaining to the effect of T. har 25-92 and T. har 29-92 isolates on the plant biomass (root length, shoot length, and fresh weight and dry weight of root and shoots)

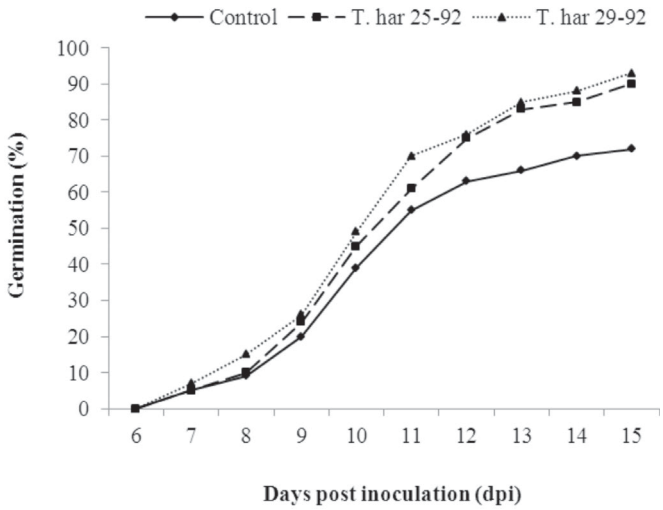

Fig. 3. Effect of *Trichoderma harzianum* isolates on the germination percentage of chili seeds in *in vitro* condition.

were showed in **Table 1**. Maximum increase in the shoot and root length (33.45 and 26.00 cm), shoot and root fresh weight (19.50 and 12.90 g) and shoot and root dry weight (3.10 and 1.87 g) was recorded for the seeds treated with T. har 29-92, and the lowest was recorded in control. The highest level of chlorophyll was observed in plants treated with T. har 29-92 (33.2 spad units) followed by T. har 25-92 (28.5 spad units) **(Table 1)**. Control plants were observed to have physiologically weak growth and less green colour when compared to both the treatments.

Table 1. Effect of plant growth promoting *Trichoderma harzianum* isolates on different growth parameters in chilli under greenhouse pot trials.

Parameter(s)	*Control*	*T. har 25-92*	*T. har 29-92*
Shoot length (cm)	23.07 ± 1.24	29.78 ± 1.22	33.45 ± 1.32
Root length (cm)	18.33 ± 1.15	21.80 ± 1.42	24.26 ± 1.24
Shoot fresh weight (g)	10.33 ± 1.05	16.33 ± 1.22	19.50 ± 1.34
Root fresh weight (g)	8.83 ± 1.04	19.70 ± 1.04	12.90 ± 1.45
Shoot dry weight (g)	2.20 ± 1.17	2.50 ± 1.34	3.10 ± 1.23
Root dry weight (g)	1.23 ± 1.50	2.00 ± 1.42	1.87 ± 0.98
Chlorophyll (spad units)	26.6 ± 1.32	28.5 ± 0.99	33.2 ± 1.34

Biocontrol assay

Biocontrol efficacy of T. har 25-92 and T. har 29-92 was evaluated in the greenhouse. In the T. har 29-92 treated plants, no disease was noticed up to 21 days, whereas, 27% and 31% disease intensity was recorded in the plants inoculated with T. har 29-92 and T. har 25-92, respectively whereas it was 79% in control **(Fig. 4)**. The root colonizing population of T. har 29-92 and T. har 25-92 was ranged between 43-74% and a gradual increment in root colonizing population was observed with progress of time **(Fig. 5)**.

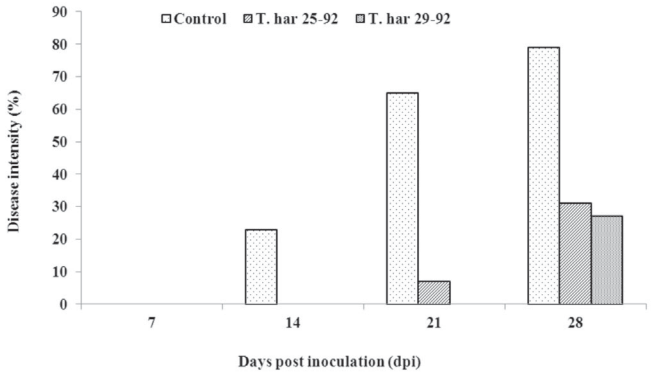

Fig. 4. Efficacy of *T. harzianum* isolates (T. har 25-92 and T. har 29-92) in reduction of *Alternaria* leaf spot disease in chilli in greenhouse trials. Control plant indicates healthy plants not inoculated with *A. solani* or *T. harzianum*.

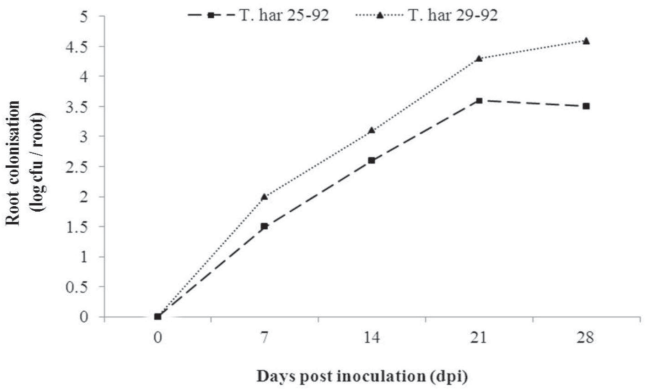

Fig. 5. Root colonization by *Trichoderma harzianum* (T. har 25-92 and T. har 29-92) in chilli plants at 0, 7, and 14 and 28 days post inoculation of *A. solani*.

Both isolates were able to colonize chili root by 3.5-4.6 log cfu plant^{-1}.

Biochemical basis of plant defense induction

Pronounced effect of *T. harzianum* application on the accumulation of phenolic contents and peroxidase activity in treated chilli plants was recorded **(Fig. 6 &7)**. Elevated accumulation (17-18%) of total phenolic contents was noticed in the leaves of chilli plants inoculated with T. har 25-92 and T. har 29-92. The peroxidase activity was also increased by 2 to 3 fold in the leaves. Maximum induction in the peroxidase and total phenolics was observed up to three days post inoculation in case of both treatments. A sharp decline in the activity of both the enzymes was recorded after 3 days post inoculation.

Inducing the plant's own defense mechanisms by prior application of biotic and abiotic inducers is the novel plant protection strategy (Kashyap and Dhiman, 2009). Fungi belonging to *Trichoderma* genus are well known as biological inducers and antagonistic toward a number of plant pathogenic fungi (Jyotsana *et al.*, 2008). There are a

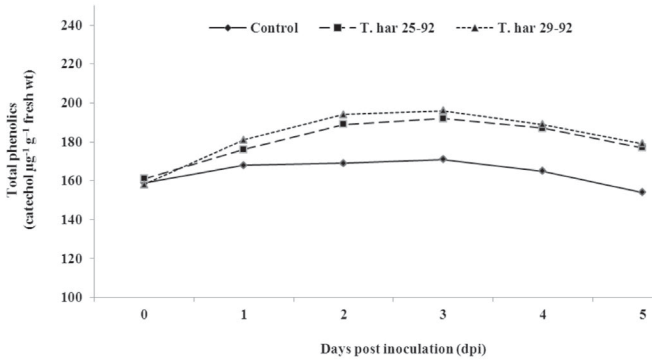

Fig. 6. Induction of total phenolics by T. har 25-92 and T. har 29-92 in chilli plants at 0, 1, 2, 3, 4 and 5 days post inoculation of *A. solani.*

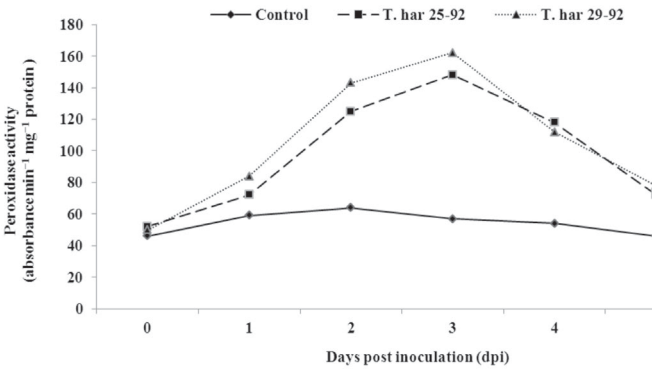

Fig. 7. Induction of peroxidase activity by T. har 25-92 and T. har 29-92 in chilli plants at 0, 1, 2, 3, 4 and 5 days post inoculation of *A. solani.*

number of mechanisms involved in *Trichoderma* antagonism namely antibiosis whereby the antagonist fungus produces antibiotics, competes for nutrients and mycoparasitism, whereas *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β-1,3 glucanases and proteases (Lorito *et al.*, 1993; Almeida *et al.*, 2007; Radjacommare *et al.*, 2010). Such hydrolytic enzymes partially degrade the pathogen cell wall that leads to parasitization (Kubicek *et al.*, 2001). The present investigation clearly demonstrated significant enhancement in levels of hydrolytic enzymes (β-1, 3-glucanse and chitinase) in comparison to the control, caused the lysis of *A. solani* hyphae. Similar observations were made by Cortes *et al.* (1998) and Howell (2003) wherein the activity of lytic enzymes (β-1, 3-glucanses and chitinases) were responsible for lysis of pathogen hyphae through digestion of major cell wall components.

Increased growth response in lettuce bean, cucumber, and pepper has been demonstrated following application of *Trichoderma* spp. under greenhouse or field conditions (Baker, 1989; Kleifeld and Chet, 1992; Ousley *et al.*, 1994; Inbar *et al.*, 1994, Yedidia *et al.*, 2001). The result presented here also demonstrate the significant increase in growth of chilli plants for each of the parameters; plant height, dry weight, chlorophyll components under greenhouse. The cumulative root and shoot biomass increased by 1.5-2.0 folds. It has been suggested that *T. harzianum* might affect plant growth as a result of their ability to influence plant hormones and vitamins (Baker, 1989; Kleifield and Chet, 1992). Such substances could influence the early stages of plant growth with better development of plant roots. The enhancement in roots and growth rate enables the plants to explore a greater volume of soil due to an increase in number of active site of uptake per unit area. Thus, they might be able to sequester more phosphate and other mineral ions liberated as a result of solublization by microorganisms. The increase in phenolic compounds and lytic enzymes exhibits the induction of defense response in the plants which is further confirmed by control of leaf spot disease in chilli plants.

The improved control of chilli leaf spot by applications of T. har 25-92 and T. har 29-92 is reflected from the enhanced activities of defense-related enzymes in chilli. The phenolic compounds may contribute to enhance the mechanical strength of host cell wall and thus may also inhibit the fungal growth (Jyotsana *et al.*, 2008; Senthilraja *et al.*, 2010). Recent reports suggested that *Trichoderma* might stimulate the production of biochemical compounds of phenolic nature associated with the host defense (Kavino *et al.*, 2008; Radjacommare *et al.*, 2010). Of these, the early induction of PAL is more important as it is the first enzyme in the phenylpropanoid pathway that leads to production of phytoalexin, phenolic substances leading to the formation of lignin with peroxidases. Conspicuously, the present study also recorded the higher activity of total phenolics and peroxidase in plants treated with T. har 25-92 and T. har 29-92 and thus generating the speculations of induced defense responses in plants treated with biocontrol agents. Thus, enhanced accumulation of phenolic compounds in chilli plants treated with *T. harzianum* is involved in reduced infection by *A. solani* and enhanced plant growth attributes. Moreover, the current study offers the scope for the effective management of chilli leaf spot in a sustainable way.

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Short Communications

Yield and quality improvement of mango through drip irrigation coupled with polyethylene mulching under high density planting

V.K. Singh, Manoj Kumar Soni and Anurag Singh

Division of Crop Production, Central Institute for Subtropical Horticulture, Rehmankhera, P.O. Kakori, Lucknow - 226101
Corresponding author: singhvk_cish@rediffmail.com

ABSTRACT

A field experiment was conducted at Central Institute for Subtropical Horticulture, Lucknow during 2005-2011 with an objective of improving the yield and fruit quality of mango cv. Dashehari through drip irrigation and polyethylene mulching under two densities 9.0 x 6.0 and 6.0 x 3.0 m, replicated three times in a randomized block design. The experimental plot of mango (six year old) was irrigated with a drip irrigation system having 4 emitters per plant of 8 liter per hour (lph) capacity based on four irrigation levels (T_1 = 40%, T_2 = 60%, T_3 = 80% and T_4 = 100% of pan evaporation). UV stabilized black polyethylene mulching (100 µ thickness) was done during October - November. Higher fruit set, fruit weight, fruits number and fruit yield per tree were recorded by application of irrigation at 60% PE per day per plant under black polyethylene mulching. Water saving to the tune of 45-58 % through mulching was recorded in all treatments. Fruit yield varied from maximum value of 5.90 t/ha in treatment T_2 as compared to control (3.22 t ha^{-1}). Drip irrigation coupled with polyethylene mulching resulted in better quality of fruits in terms of increased TSS (25.33 ^{o}Brix), firmness (0.68 kg cm^{-2}) and reduced cumulative physiological losses (5.45%) under 9.0 x 6.0 m density. Whereas under 6.0 x 3.0 m the treatment T_3 (80% PE) showed maximum value in terms of yield (12.82 t/ha), TSS (25.13 ^{o}Brix), firmness (0.74 Kg cm^{-2}) and reduced cumulative physiological losses (6.09 %). Thus, drip irrigation at 60% PE and 80 % PE level coupled with black polyethylene mulching improves the yield and quality of mango *var*. Dashehari under 9.0 x 6.0 m and 6.0 x 3.0 m densities, respectively.

Keywords: Mango, Dashehari, drip irrigation, mulching, yield, quality.

INTRODUCTION

The economic development of country is dependent on the horticulture production because it provides more revenue per hectare in addition to nutritional security. Despite the exponentially growing population, food production is limited by land and water. Irrigation is a very important factor impacting growth, yield and quality of any crop. Mango is widely grown in India, whose production reached 15.03 million tones which amounts to 21% of the total fruit production. Uttar Pradesh contributes to 23.9% in mango basket of the country. Although growing areas in India have shown increasing trend but the mean fruit yield has plateaued to nearly 6.5 tonnes/ha (NHB Database, 2010). As the water resources are becoming scarce day by day, appropriate water utilization must be ensured by adopting efficient irrigation management practices. It is observed that the application of a higher irrigation water volume does not result in higher yields of fruits (Azevedo *et al*., 2003; Gonzalez *et al*., 2004; De bie, C.A.J.M., 2004). The empirical approach on irrigation of several subtropical and tropical fruit orchards suggests that if the amount of irrigation water is excessive it could lead to the leaching of applied nutrients and pesticides into the groundwater (Schaffer, 1998). Moreover, the surplus water may increase soil salinity and groundwater contamination. Irrigation scheduling based on empirical value of crop coefficient greatly influences production cost, crop yield and fruit quality. Intensive knowledge of the changes in water levels and its association with evapotranspiration in mango is essential to improve upon the yield and fruit quality limiting factors (Zhang *et al*., 2004; Allen *et al*., 1998). The present paper examines the impact of drip irrigation coupled with mulching on mango *cv*. Dashehari orchard for improving yield and quality, grown in a subtropical environment based on four irrigation levels.

MATERIALS AND METHODS

A field experiment was conducted at Central Institute for Subtropical Horticulture, Lucknow with an objective of improving the yield and fruit quality of mango *cv*. Dashehari through drip irrigation and polyethylene mulching with two densities 9.0 x 6.0 m (185 tree ha^{-1}) and 6.0 x 3.0 m (555

tree ha^{-1}), replicated three times in a randomized block design. The experimental plot of mango (six year old), having sandy loam texture, was irrigated with a drip irrigation system having 4 emitters per plant of 8 lph capacity in 9.0 x 6.0 m density and 2 emitters of same capacity per plant in 6.0 x 3.0 m density. The experiment has four irrigation levels with drip system (40%, 60%, 80% and 100% PE replenishment), one basin irrigation with 100% PE replenishment under mulch and non-mulch conditions making ten treatments in all. The drippers were placed equidistant at 50% distance of canopy radius. The water received though rain was accommodated in irrigation schedule in successive days in all treatments but neglected in control plants. The water requirement was determined by multiplying the canopy area (m^2 x pan evaporation (mm) x evaporation replenishment (%). One square meter canopy area with one ml evaporation replenishment was equal to one liter of drip irrigation water (Dinesh *et al*., 2008). The time of drip operation was determined by the total quantity of water required in a litre divided by total discharge rate. UV stabilized black polyethylene mulching (100 micron thick) was done during October-November. Observations on meteorological parameters *viz*., pan evaporation, temperature, relative humidity and plant growth *viz*., height (m), yield (kg plant^{-1}), fruit weight (g), and quality attributes *viz*., TSS (^{o}Brix), firmness (kg cm^{-2}) and cumulative physiological loss of weight (CPLW) (%) at appropriate stages were recorded.

RESULTS AND DISCUSSION

In present study, among two types of plantation of mango *cv*. Dashehari, the 9.0 x 6.0 m density of plantation was better than 6.0 x 3.0 m in terms of quality fruit production per plant **(Table 1&2)**. Drip irrigation 60% PE level coupled with black polyethylene mulching (T_2) showed significantly

Table 1. Effect of drip irrigation and polythene mulching on tree height, yield and fruit quality of mango *cv*. Dashehari under 9.0 x 6.0 m density

Treatment	*Details*	*Height (m)*	*Yield (t ha^{-1})*	*Fruit weight (g)*	*TSS (^{o}Brix)*	*Firmness (kg cm^{-2})*	*CPLW (%)*
T_1	40% PE + PM	2.87	4.36	210.20	25.33	0.68	6.12
T_2	60% PE + PM	3.03	5.90	197.48	25.80	0.84	5.45
T_3	80% PE + PM	2.72	5.29	195.64	26.13	0.74	6.19
T_4	100% PE + PM	2.61	4.18	211.20	25.73	0.82	6.42
T_5	40% PE + WPM	2.50	3.10	160.82	24.66	0.65	6.52
T_6	60% PE + WPM	2.88	4.71	166.20	24.73	0.67	6.46
T_7	80% PE + WPM	2.60	4.25	165.75	25.20	0.61	6.73
T_8	100% PE + WPM	2.97	3.42	169.46	24.00	0.60	6.85
T_9	100% PE basin irrigation + PM	2.79	3.22	171.53	24.53	0.57	6.99
T_{10} (Control)	100% PE basin irrigation + WPM	2.68	2.72	162.30	24.26	0.54	7.07
CD (p=0.05)		N.S.	0.218	17.395	3.61	0.178	0.338

PE- Pan evaporation, PM- Mulched, WPM- Unmulched

Table 2. Effect of drip irrigation and polythene mulching on tree height, yield and fruit quality of mango *cv*. Dashehari under 6.0 x 3.0 m density

Treatment	*Details*	*Height (m)*	*Yield (t ha^{-1})*	*Fruit weight (g)*	*TSS (^{o}Brix)*	*Firmness (kg cm^{-2})*	*CPLW (%)*
T_1	40% PE + PM	2.67	11.01	185.77	24.27	0.63	6.13
T_2	60% PE + PM	2.65	11.98	184.07	23.87	0.62	6.06
T_3	80% PE + PM	2.63	12.82	196.64	25.13	0.74	6.09
T_4	100% PE + PM	2.61	11.68	196.11	25.53	0.68	5.48
T_5	40% PE + WPM	2.62	9.45	185.50	24.27	0.58	6.34
T_6	60% PE + WPM	2.63	9.96	181.87	23.07	0.61	6.26
T_7	80% PE + WPM	2.53	10.46	185.32	23.33	0.71	6.45
T_8	100% PE + WPM	2.79	11.62	175.05	22.80	0.57	6.69
T_9	100% PE basin irrigation + PM	2.60	9.14	181.03	21.80	0.52	7.16
T_{10} (Control)	100% PE basin irrigation + WPM	2.58	7.29	178.83	21.33	0.48	7.31
CD (p=0.05)		0.355	0.869	12.076	2.257	0.092	0.284

PE- Pan Evaporation, PM- Mulched, WPM- Unmulched

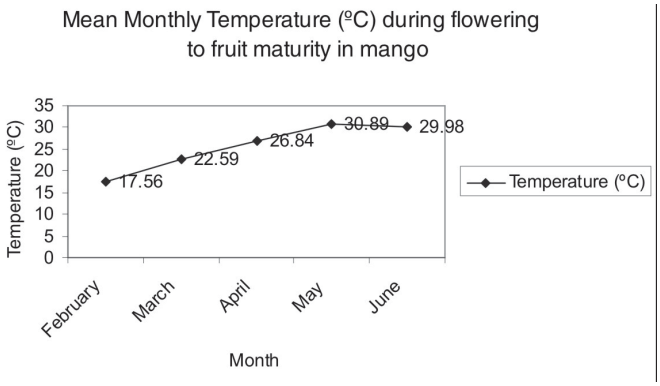

Fig. 1. Mean monthly temperature (2010-11, 11-12) during flowering and fruiting in mango *cv.* Dashehari

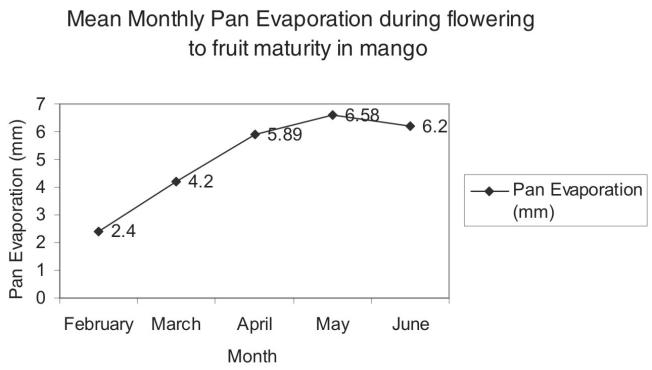

Fig. 2. Mean monthly pan evaporation during flowering to fruit maturity in mango *cv.* Dashehari

high yield 5.90 t ha^{-1} and quality in terms of TSS (25.8 ^{o}Brix), firmness (0.84 kg cm^{-2}) and reduced cumulative physiological losses (5.45%) under 9.0 x 6.0 density planting as compared to minimum yield (3.22 t ha^{-1}), TSS (24.53 ^{o}Brix), firmness (0.57 kg cm^{-2}) and high cumulative physiological losses (7.07 %) in control trees. On the other hand, under high density planting 6.0 x 3.0 m, 80% PE level coupled with black polyethylene mulching (T_3) resulted in higher yield (12.83 t ha^{-1}) as well as quality of fruits in terms of improved TSS (25.53 ^{o}Brix), firmness (0.74 kg cm^{-2}) and reduced cumulative physiological losses (5.48%) at ripening of fruit as compared to less TSS (21.33 ^{o}Brix), low firmness (0.48 kg cm^{-2}) and more CPLW (7.31%) in untreated control trees. Water saving to the tune of 56.82 to 60.07% through mulching was recorded under mulched trees as compared to unmulched trees (39.7 to 42.05%) **(Fig. 4)**.

On perusal of the data it was found that the relative humidity showed consistently high negative correlation with pan evaporation **(Fig. 2&3)**. The soil was sandy loam in texture having high organic carbon, P, K, Ca in 0-25 cm soil depth under both densities **(Table 3)**. The mean air temperature also showed consistently higher positive correlation with pan evaporation **(Fig. 1 & 3)**. In case of drip irrigation water is made available in the root zone thereby reducing the water stress pressure near roots (Bankar *et al.*, 1993). Water required for drip irrigation was lower than that of surface irrigation. The irrigation was applied on alternate days during summer season and twice a week during winter

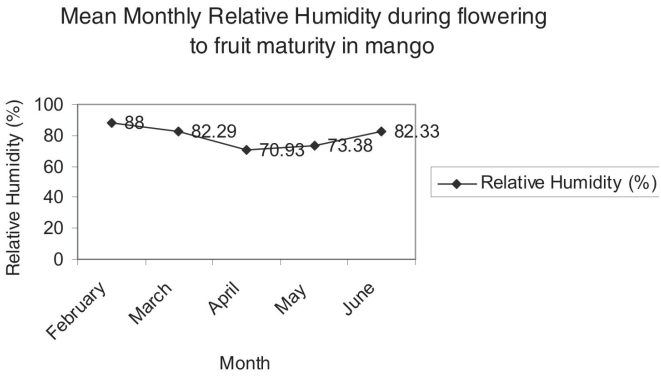

Fig. 3. Mean monthly relative humidity during flowering to fruit maturity in mango *cv.* Dashehari

season. The irrigation was applied at 0-25 cm of soil depth as the layer showed high variation in soil moisture **(Table 4)**. Similar results were also reported by Panigrahi *et al.* (2010), Ghosh and Bauri, (2003) and Dixit *et al.* (2003) in mango and Shirgure *et al.* (2004) in citrus.

Table 4. Soil moisture content at different depths under mango trees

Treatment	*Depth. (cm)*	*Moisture(%)*
Mulched	0-25	30.6
	25-50	31.4
	50-100	14.1
Unmulched	0-25	15.8
	25-50	22.5
	50-100	14.6

Table 3. Soil characteristics of mango under different densities

Density (m)	*Depth. (cm)*	*pH*	*Organic carbon %*	*Avail. P (ppm)*	*Avail. K (ppm)*	*Exchg. Ca (ppm)*	*Cl (mg 100g)*
3m x 6m	0-25	6.94	0.350	6.0	87.5	128.8	8.8
	25-50	6.97	0.311	6.0	81.8	117.5	8.8
	50-100	6.99	0.233	4.7	82.5	117.5	6.6
6m x 9m	0-25	6.97	0.331	6.7	76.2	119.3	8.8
	25-50	6.77	0.272	5.3	71.2	113.7	6.6
	50-100	6.74	0.253	4.7	75.0	117.5	6.6

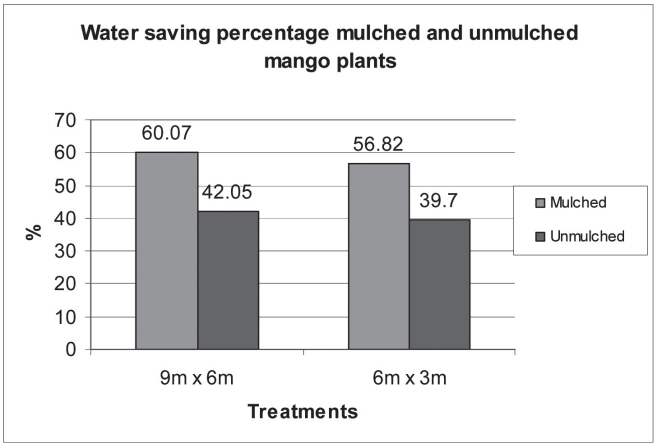

Fig. 4. Water saving percentage mulched and unmulched mango plants under 9m x 6m and 6m x 3m density in mango cv. Dashehari

It is concluded that mango should be irrigated at 60% PE coupled with polythene mulching in 9 x 6 density of plantation and 80% PE coupled with polythene mulching in 6 x 3 density planting. Increasing the amount of irrigation above the threshold limit (60% PE) affects production adversely.

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Evaluation of bio-efficacy of *Pochonia chlamydosporia* (*Verticillum chlamydosporium*) 1% W. P. against *Meloidogyne incognita* on okra in different agro-climatic regions

M. S. Rao, Kusum Dwivedi, S. P. Tiwari, M. K. Chaya, R. Manoj Kumar and T. N. Shivananda

Indian Institute of Horticultural Research, Hessaraghatta Lake P. O., Bangalore 560 089, India
Department of Entomology, C.S. Azad University of Agric. & Tech. Kanpur (U.P)
Department of Plant Pathology, Jawahar Lal Nehru Krishi Vishva Vidyalaya, Jabalpur (M.P)
Corresponding author: msraobio45@gmail.com

ABSTRACT

Field studies at multi-location sites of various agro-climatic regions of India were conducted to assess the efficacy of bio-nematicide - *Pochonia chlamydosporia* 1% W.P. Zare *et al*., (*Verticillum chlamydosporium* Goddard) in the management of *Meloidogyne incognita* (Kofoid *et* White) Chitw on Okra (*Abelmoschus esculentus* Moench.). These experiments clearly indicated the potential of this bio-nematicide in the management of *M. incognita* on okra. In these investigations *P. chlamydosporia* was enriched in farm yard manure (FYM) and applied at different doses in the experimental plots. Results indicated that the seed treatment @ 20g *P. chlamydosporia*/kg and application of 5 tons of FYM enriched with 5 kg *P. chlamydosporia*/ha proved to be significantly effective in reducing the population of *Meloidogyne incognita* and increasing the yield of okra in these agro-climatic regions.

Keywords: *Pochonia chlamydosporia*, FYM enrichment, management, agro-climatic regions, *Meloidogyne incognita*, Okra

INTRODUCTION

Root-knot nematode (*Meloidogyne incognita*) is reported as one of the economically important pests of okra (*Abelmoschus esculentus* Moench) causing 30-50% yield reduction (Bhatti and Jain, 1977). The nematode makes the plant vulnerable to attack by *Fusarium oxysporum* f.sp. *vasinfectum* (Abuzar and Haseed, 2006). Reports on hazards of indiscriminate use of agro-chemicals and importance of sustainability in crop production necessitated the need to develop a pragmatic management strategy, using FYM enriched with *Pochonia chlamydosporia* which was reported to be very promising bio-nematicide by various researchers (De Leij and Kerry, 1991; Kerry *et al*., 1993; Reddy *et al*., 1999; Dhawan and Singh, 2010).

This bio-nematicide is being used for seed treatment at dosages ranging from 5 to 20g/kg of seed by different institutions. A dosage of 10g of above mentioned bio-nematicide for treating 1kg of seed of okra or any other vegetable crops is recommended (Rao *et al*., 1997; 2003 and Reddy *et al*., 1999). Subsequently many farmers complained ineffectiveness of this dosage in okra and other vegetable crops. That made us to re-look into the practice of the farmers how they handled the bio-pesticides. After analyzing actual problems faced by the farmers we made efforts to investigate effects of different dosages of *P. chlamydosporia* 1% W.P. for the seed treatment in the experiments for management of *M. incognita* at different agro-climatic regions such as in Karnataka, Uttar Pradesh and Madhya Pradesh.

MATERIALS AND METHODS

Multi-location sites selected for the study in India were at Indian Institute of Horticultural Research (IIHR), Bengaluru (Karnataka), C. S. Azad University of Agriculture & Technology, (CSAUA&T), Kanpur (Uttar Pradesh) and Jawahar Lal Nehru Vishwa Vidyalaya (JNKVV), Jabalpur (Madhya Pradesh). A local isolate of *P. chlamydosporia* (the identification confirmed by Professor Brian Kerry, Rothamsted Research, United Kingdom) was mass produced at IIHR, Bengaluru.

Initially to determine appropriate dose for seed treatment, seeds of okra (cv. Arka anamika) were treated at the rate of 5g/ 10g/ 20g/kg alongwith control at a room temperature of 27 ºC and a relative humidity of 56.4%. Five samples (5 replicates) of one gram of seed from all the treatments were taken and analyzed for adhesion of *P. chlamydosporia* propagules to the surface of seeds which was expressed as colony forming units (CFU/gram of seeds). CFUs were analyzed through standard serial dilution technique using semi-selective medium of *P. chlamydosporia* (Kerry *et al*.,

1993). Further, okra seeds treated with 5g/ 10g/ and 20g/ kg were also sown in soil filled in pots. Observations were recorded on colonization of roots by bio-agents in 30 day old okra plants. To evaluate the colonization by the root system was carefully washed to remove soil, blotted dry, weighed and cut into small pieces of about 1 cm each. One gram samples of roots were taken at random and root colonization by *P. chlamydosporia* was assessed by using the semi-selective medium developed by Kerry *et al*. (1993). The Petri plates were incubated at 25-27 ˚C for 15 days. To study egg parasitism, 20 egg masses from each replicate, were randomly selected and treated with 0.05% sodium hypochlorite and the number of eggs infected were counted under microscope. *P. chlamydosporia* was isolated from adult females and eggs of *M. incognita* by using the semi-selective medium mentioned above.

For the field experiments formulated product of bio-nematicide *P. chlamydosporia* 1% W.P. (CFU 2 x 10^6/gram) was added to FYM and left under shade for 15 days at moisture content of 25-28%. The experiments were conducted at the different agro climatic regions such as IIHR, Bengaluru, Karnataka (13°58'N, 78 E, and at 890m above sea level), C.S. Azad University of Agriculture & Technology Kanpur (25°26' to 26°58' N, 79°31' to 80°34' E and 125.9 m above sea level) and Jawahar Lal Nehru Krishi Vishva Vidyalaya, Jabalpur. Experiments at IIHR were conducted during 2006 - 2009 with a minimum prevailing temperature ranging from 15.8 ˚C – 21.5 ˚C and maximum temperature ranging 27.1 ˚C – 34.1 ˚C. Experiments at Kanpur were conducted during 2008-2009 with a minimum prevailing temperature ranging from 5 ˚C – 28 ˚C and maximum temperature ranging 16 ˚C –. 42 ˚C (Madhya Pradesh) is geographically located at 22°49' and 20°80' N, 78°21' and 80°58' E longitude and at an altitude of 411.78 meters above the sea level. Experiments at JNKVV, Jabalpur were conducted during 2008-2009 with a minimum prevailing temperature ranging from 4 ˚C – 29 ˚C and maximum temperature ranging 8 ˚C – 43 ˚C.

The experimental fields were divided into 4 x 2.5 m plots. Initial population densities of nematode in soil at all the centres were in the range of 136-152 J_2 per 100 g of soil. The treatment of seeds at the rate of 20g /kg was found to be significantly effective than other dosages, and so only this treatment (20g/kg) was included in the field trials at different agro-climatic regions. The soil in the experimental fields was incorporated with bio-nematicide enriched FYM and un-enriched FYM and the seven treatments included different combinations of seed treatment along with bio-nematicide enriched and un-enriched FYM along with one control without any treatment **(Table 2)**.

All the treatments were replicated ten times in a randomized block design. After, three months of sowing of okra, data on root-knot index on a 1-5 scale of Bridge and Page, (1980), nematode population densities in roots and percent eggs parasitization by the bio-agent were recorded. To study egg parasitism, 20 egg masses from each replicate, were randomly selected and treated with 0.05% sodium hypochlorite and the number of eggs infected were counted under microscope. *P. chlamydosporia* was isolated from adult females and eggs of *M. incognita* by using the semi-selective medium developed by Kerry *et al*. (1993). The experiments were conducted in two seasons during 2006-2009 in all these agro-climatic regions. The data were analyzed by using standard statistical methods.

RESULTS AND DISCUSSION

Treatment with 20g *P. chlamydosporia* 1% W.P. per 1 kg of okra seeds resulted in significantly higher levels of adhesion of propagules of *P. chlamydosporia* **(Table 1)**. Further, there was significantly higher colonization of *P. chlamydosporia* on roots of okra seedlings which were raised by treating the seeds @ 20g/ kg **(Table 1)**. Hence, in subsequent experiments the seeds treated at the rate of 20g of *P. chlamydosporia* 1% W.P. per one kg of seed of okra were alone used.

Table 1. Effect of seed treatment with *P. chlamydosporia* 1% W.P. (Bengaluru).

Treatments	*Adhesion of propagules of P. chlamydosporia to seed (CFU/g of seed) x10^5*		*Root – colonization of P. chlamydosporia (CFU/g of root) x10^5*	
	I season	*II season*	*I season*	*II season*
5g/ kg	1.3	2.4	2646	3231
10g/ kg	1.8	3.2	3832	4328
20g/ kg	4.2	6.7	5679	5983
Control	0	0	0	0
CD 5%	0.84	0.84	1.21	1.21

The results of the field trials indicated significant reduction in the root-knot index on okra roots in the treatment where seeds were treated @ 20 g/kg and incorporated 5 tons of FYM enriched with 5 kg of bio-nematicide *P. chlamydosporia* – 1% W.P. at all the centers mentioned above **(Tables 2-4)**. This treatment has significantly increased the yield of the crop and is at par to chemical treatment (chemical check) **(Tables 2-4)**. The data on the bio-agent and its parasitization of the nematode eggs indicate that the strain of *P. chlamydosporia* is rhizosphere competent. Earlier experiments with the application of *P. chlamydosporia* clearly indicated the requirement of huge quantity of this bio-nematicide formulation to the tune of 20-25 kg/ha (Chaya and Rao, 2012). It would be very expensive for the farmer to use this huge quantity of bio-

nematicide and it is practically not possible to adopt the technology. Hence these investigations were carried out to develop a delivery system for the application of this potential bio-nematicide in different agro-climatic regions in India. FYM was found to be the most suitable organic material which could be enriched with this bio-nematicide for the application in the main field conditions.

Egg parasitization by this bio-agent was observed to increase with the increased dose of application of bio-nematicide enriched FYM **(Tables 2-4)**. Increased parasitization of eggs is a pre-requisite for the successful management of this nematode on any crop. *P. chlamydosporia* was reported to parasitize the eggs and egg masses of the root-knot nematodes (De Leij and Kerry, 1991; Kerry *et al*., 1993; Reddy *et al*., 1999). The dosage of 5 tons of FYM enriched with 5 kg of *P. chlamydosporia* – 1% W.P. was significantly effective in reducing the root-knot index and nematode population in the roots **(Tables 2-4)**. At this dosage, the bio-nematicide is significantly effective in the management of the root-knot nematodes on okra when compared to the lower dosages **(Tables 2-4)**. Un-enriched FYM application was not effective in the management of nematodes on okra, **(Tables 2-4)**. The experiments were repeated in two seasons during the year 2006 to 2009 to confirm the results on the efficacy of application of this bio-nematicide enriched FYM on the management of root-knot nematodes on okra under the field conditions at different agro climatic regions given in the text. Pooled data of two seasons are presented in the tables. The data of these field experiments indicate the potential use of *P. chlamydosporia* 1% W.P. enriched FYM for the bio-management of *M. incognita* on okra under field conditions.

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The authors thank the Director of IIHR, Bangalore, Directors of Research of C.S.Azad University of Agriculture & Technology, Kanpur (Uttar Pradesh), Jawahar Lal Nehru Krishi Vishva Vidyalaya, Jabalpur (Madhya Pradesh), for facilitating this research.

Table 2. Effect of *P. chlamydosporia* (*V. chlamydosporium*) – 1% W.P. on *M. incognita* and yield of Okra at IIHR, Bengaluru.

Treatments	*Root Knot index*	*Production per plot (kg)*	*Yield q/ha*	*% increase in the yield of the crop over control (per ha)*	*Number of M. incognita in 10g of roots*	*% egg parasitized by V. chlamydosporium (P. chlamydosporia)*
T_1	2.8	9.95	99.5	2.4(8.91)	25	08(16.43)
T_2	2.4	10.53	105.3	8.4(16.45)	20	22(27.97)
T_3	2.2	10.97	109.7	12.9(24.50)	17	46(42.71)
T_4	1.6	11.39	113.9	17.2(24.50)	12	62(51.94)
T_5	3.0	9.89	98.9	1.8(7.71)	26	0(0)
T_6	1.5	11.03	110.3	13.5(21.56)	15	0(0)
T_7	3.2	9.72	97.2	0	29	0(0)
C.D 0.05	0.28	1.69	5.22	0.48(4.05)	1.43	3.43(10.63)

Figures in parenthesis are angular transformed values.

T1: Seed treatment with *P. chlamydosporia*– 1% W.P. @20g/kg of seed; T2: T1 + application of 5 tons of FYM enriched with 2kg of *P. chlamydosporia* – 1% W.P. /ha; T3: T1 + application of 5 tons of FYM enriched with 3.5kg of *P. chlamydosporia* - 1% W.P. /ha; T4: T1 + application of 5 tons of FYM enriched with 5kg of *P. chlamydosporia* - 1% W.P. /ha; T5: Application of 5 tons of un-enriched FYM /ha. T6: Carbofuron application @ 1 kg a.i./ha; T7: Control without any treatment.

Table 3. Effect of IIHR- *V. chlamydosporium* (*P. chlamydosporia*) – 1% W.P. on *M. incognita* and yield of Okra at CSAUA&T, Kanpur

Treatments	*Root Knot index*	*Production per plot (kg)*	*Yield q/ha*	*% increase in the yield of the crop over control (per ha)*	*Number of M. incognita in 10g of roots*	*% egg parasitized by V. chlamydosporium (P. chlamydosporia)*
T_1	2.8	6.27	62.7	2.0(8.13)	15	6(14.181)
T_2	2.4	6.34	63.4	3.2(10.31)	12	31(33.83)
T_3	2.2	6.59	65.9	7.3(15.68)	9	55(47.87)
T_4	1.6	6.73	67.3	9.4(17.85)	6	64(53.13)
T_5	3.0	6.24	62.4	1.5(7.04)	16	0(0)
T_6	1.5	6.61	66.1	7.5(15.89)	8	0(0)
T_7	3.2	6.15	61.5	0(0)	17	0(0)
C.D 0.05	0.54	1.26	5.48	0.95(5.59)	1.65	2.47(9.10)

Figures in parenthesis are angular transformed values.

Table 4. Effect of IIHR- *V. chlamydosporium* (*P. chlamydosporia*) – 1% W.P. on *M. incognita* and yield of Okra at JNKVV, Jabalpur

Treatments	*Root Knot index*	*Production per plot (kg)*	*Yield q/ha*	*% increase in the yield of the crop over control (per ha)*	*Number of M. incognita in 10g of roots*	*% egg parasitized by V. chlamydosporium (P. chlamydosporia)*
T_1	4.28	7.81	78.1	2.5(9.10)	27	10(18.44)
T_2	3.75	8.03	80.3	5.4(13.44)	21	37(37.47)
T_3	3.27	8.45	84.5	11.0(19.37)	18	52(46.15)
T_4	2.50	8.82	88.2	15.7(23.34)	13	61(51.35)
T_5	4.76	7.75	77.5	1.8(7.71)	28	0(0)
T_6	2.26	8.57	85.7	12.5(20.70)	14	0(0)
T_7	5.0	7.62	76.2	0(0)	30	0(0)
C.D 0.05	0.87	1.49	4.56	1.43(6.80)	2.76	3.59(10.94)

Figures in parenthesis are angular transformed values.

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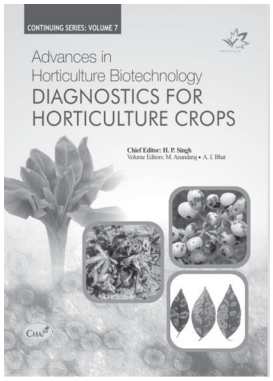

Advances in Horticulture Biotechnology (Vol. 7)
DIAGNOSTICS FOR HORTICULTURE CROPS

(CONTINUING SERIES VOLUME 7)

H. P. Singh, M. Anandaraj and A. I. Bhat

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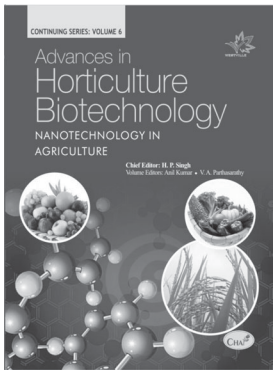
Plant diseases impose serious limitations on cultivation of crops globally more so in the perennial horticultural crops. Unlike in animals, plant disease diagnostics research and development have received very little attention except for a few virus diseases. There is huge scope for development and deployment of plant diagnostic tools to produce healthy planting materials. Production of quality planting materials is fast becoming an important input in disease management programmes especially in production of disease/virus free plants. Immuno-assays and nucleic acid based diagnostic techniques occupy the main positions as methods of diagnosis in the contemporary plant pathology. Among them, PCR and its modifications as well as various ELISA formats are most popular. Serology and PCR based diagnostics have been developed for several pathogens infecting different horticultural crops. While some of the diagnostic assays have been validated and are being used in routine large scale indexing, a few of them still needs validation. There is a need for awareness creation on the importance of disease-free planting material and capacity building of all stakeholders.

The volume on **Diagnostics for Horticulture Crops** attempts to provide insight into the progress made in the diagnostics of pathogens infecting various horticultural crops and identify the gaps and future thrust areas. The volume contains 19 chapters on different horticultural crops including fruits, vegetables, plantations crops and spices, and orchids. Each chapter, written by experts of the crops, is exhaustive review of different diagnostic methods including biological, physical (morphological), serological and nucleic acid based for all important pathogens infecting a particular crops. There is one chapter exclusively devoted on the principles and methodologies of different diagnostic techniques currently available for plant pathogen detection. References to the literature cited have been indicated throughout the text to enable a reader to obtain more information on the subject of interest. Very eminent scientists working on diagnostics of different horticultural crops have contributed the chapters. The Chief editor Dr HP Singh has been editing a series of volumes in Advances in Horticultural Biotechnology and this volume is unique as it covers an entirely new field of emerging science.

Overall, this book is an excellent and comprehensive compilation of diagnostics available for different pathogens infecting horticultural crops. I hope this volume will serve as an excellent reference book for research workers, teachers, extension workers, students and anybody who is interested in diagnosis of plant diseases.

Prof. K.M.L. Pathak
Deputy Director General (AS)
Indian Council of Agricultural Research
New Delhi



Advances in Horticulture Biotechnology (Vol. 6) NANOTECHNOLOGY IN AGRICULTURE

(CONTINUING SERIES VOLUME 6)

H. P. Singh, Anil Kumar, V.A. Parthasarathy and Babita Singh
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Nanotechnology is currently one of the fastest growing technical fields that involve the design, characterization, production and application of structures, devices and systems by controlled manipulation of size and shape at the nanometer scale (atomic, molecular and macromolecular scale) that produces structures, devices and systems with at least one novel/superior characteristic or property. Nanotechnology has enormous applications in every walk of life including biomedical, veterinary and agriculture. To address the emerging challenges faced by modern agriculture, there is a need of technologies for enhancing the efficiency, improving the quality and reducing the wastages. Nanotechnological interventions which include nano-particles based disease diagnostics, nano-insecticides for insect-pest control, nano-formulations for nutritional studies and allelopathy and various other aspects will certainly enhance the efficiency and precision in agriculture. Western countries like USA, Germany etc., and even China is much ahead in applying nanotechnology for enhancing agriculture production. Efforts are also being made in our country to harness the potential of nanotechnology for boosting the production.

Considering the important role of nanotechnology in agriculture and to consolidate the efforts of Indian scientists, the book entitled **"Nanotechnology in Agriculture"** under the series of "Advances in Horticulture Biotechnology" is edited by Dr H. P. Singh as Chief Editor, Dr Anil Kumar, Dr V.A Parthsarathy and Dr Babita Singh as editor. The book has the highlights of Nano-diagnostics, Nano-delivery, Nano-packaging and Nano-safety. The different chapters in the book are grouped into nine sections written by expert researchers, who are at the forefront in their respective fields and belong to agri-horticulture and animal/fisheries sciences. This book covers the themes that have emerged from various dialogues during brainstorming session and accordingly chapters are contributed in each section.

Development and synthesis of the nanomaterials as well as application of nanotechnology in crop science, animal and fisheries science is discussed in section I. Section II describes the nano-sequencing approaches for detection of genetic variation which is a major task for plant breeders. Section III includes Nanosensors or nano-scale, wireless sensors that represent the intersection of nanotechnologies and information technologies while section IV discusses the nano-particles based disease diagnostics and control. Section V describes an application of nanotechnology related to nano-insecticide for insect pest control. Section VI covers the real challenge of feeding burgeoning population by nanoformulations for nutritional studies and allelopathy. Section VII describes the problems faced in post harvest management which includes post harvest handling, post harvest storage etc. Section VIII introduces the application of nanotechnology in enhancing nutrient uptake that is largely restricted to crops, i.e. fertilizers. Section IX concentrates on regulations to assure that molecular machines and their products are developed in a safe and responsible manner. A chapter also deals with issues on intellectual property rights protection and non-commercial laws (environmental laws or privacy rights).

All the chapters given in each section are written in lucid manner and are thought provoking for developing new ideas and concepts. The theme of the book is pertinent with current scenario of applications of frontier sciences in agriculture. This book illustrates the basic principles and practices with examples of recent work and how the work is advancing scientific knowledge and technological capabilities. The book deals with an incredibly diverse aspect that will help in enhancing nanotechnology research and development useful for advancing a new era of industrial and agricultural technologies. Reading this book will shatter the monolithic term ''nanotechnology'' into the myriad of facets that it really is. This book therefore aims to be an informative textbook which could be helpful for anyone who is interested in its use for teaching and research related to agricultural/horticulture/animal/fisheries sciences.

Dr M. M. Pandey
Deputy Director General (Ag. Engg.)
Indian Council of Agricultural Research
New Delhi

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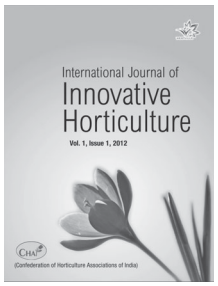

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