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Research Articles

Short Communications

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Research Articles

Pesticide-free plasticulture for sustainable vegetable production in the context of climate change

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ABSTRACT

Among plethora of insect-pests and diseases, sucking pests and related plant viruses are number one cause of concern for majority of farmers raising crops either in field or inside net/poly/greenhouse (GH) or tunnels. To explore the possibility of "zero-pesticide' plasticulture in the tropical areas, studies were conducted to investigate the effect of ultraviolet (UV) blocked greenhouses made from combination of net and plastics on the immigration of thrips (*Ceratothripoides claratris*) and occurrences of capsicum cholorosis virus (CaCV-AIT) as a model. Using UV-blocking nets and plastics, to allow ventilation – a pre-requisite for tropical greenhouses, various levels of ambient UV intensity were achieved under test greenhouses and followed so that immigration of the model pests and occurrences of virus were observed. Significantly fewer thrips immigrated under GH with lower UV intensity and consequently caused lower levels of leaf damage clearly indicating crucial role of UV in flight and forging behaviour of thrips. During open gates experiments, a total of 96-100% virus infestation was recorded under non UV-blocking greenhouses compared to 6-10% under UV-blocking greenhouses, having majority of the virus infected plants tested positive for the tospovirus, CaCV-AIT. The virus spreads were remarkably delayed for several days under greenhouses with lower UV light. These results suggests that greenhouses made from the combination of the UV-blocking nets and plastics have a significant influence on both the immigration and virus spread vectored by thrips. The results are discussed in context of pesticide free management of other sucking pests and virus spread in tomatoes under plasticulture in the context of climate change.

Key words: *Ceratothripoides claratris*, tospovirus, UV-blocking nets and plastic films, plasticulture

INTRODUCTION

Vegetable cultivation under plasticulture structures are on rise in all continents, including many countries in Asia, to raise quality and high-value horticultural crops. A range of vegetable crops including cucumber, melon, tomatoes and capsicum are widely grown in such structures. However, unlike the open-field production systems, which represents an open ecosystem, plasticulture based production system represents closed and human managed production systems, where growers have a higher degree of control on various parameters of the micro-climate.

The sucking insects-pests (Hemiptera: Suborder Homoptera) represented by whiteflies, thrips, jassids, aphids are some of commonly occurring vectors for vegetable crops responsible for transmitting some of the deadliest plant viruses. The climate change mediated variations in temperature rainfall along with anthropogenic reasons of round-the-year cultivation, increased trade related movements resulted in unprecedented increase in incidence of plant virus diseases.

Use of chemical pesticides remains a majority option for sucking pest and other insect-pest management with its well documented and known adverse impacts on overall environment. Rampant uses of chemical pesticides are directly attributed to the rapid increase in resistance, resurgence and secondary pest outbreaks (Perkins, 1982). In summary, the region is facing challenges of over use and misuse (Srinivasan, 2008), inappropriate labeling (EJF, 2002), serious quality issues, environmental pollution, health hazards to farmers and consumers (Mancini *et al*, 2009) related to the pesticides in the Asian region. In addition, the greater awareness among consumers in urban communities of the dangers of toxic residues on vegetables has created an ever growing demand (e.g. GAP) for vegetables free of pesticide residues across the region creating favorable to produce and consume healthy vegetables. In line with the FAO Codex Alimentarius, governments in many Asian countries have established Maximum Residue Levels (MRLs) for pesticides on imported vegetables (Ketalaar and Kumar, 2012).

To overcome negative externalities and improve quality, a number of efforts to promote plant-based, microbial based and other strategies have been developed (Kumar *et al*., 2005; Kumar and Poehling, 2007) but only with limited and sporadic success (Lewis, 1997). Further, the challenges of brining 'system perspective of sustainable pest management' remains an allusive dream due to 'linearity' in planning pest management. Few attempts have been made till-date to manipulate the basic behavior of the insects that have the strong potential to be part of developing a system-based pest management approach.

Many herbivorous insects studied have two or three types of photosensitive receptor cells in their compound eyes; hence they show a di- or tri-chromatic system able to differentiate not only brightness but colors by interpolating the different stimuli from the multiple receptor system for aphid vision (Döring and Chittka, 2007). However, sensitivity maxima are quite different from vertebrates. Most insects react especially to wavelength between 500 and 600 nm (green), around 450 nm (blue) and 350 – 380 nm (UV A). The function of UV sensitivity is only partly explained today. It is known that UV reception is used for inducing flight activity ("take-off"), spatial orientation during distance flights (e.g. whiteflies, thrips, aphids) as well as location of specific targets that intensively reflect light in the UV range, such as specific structures in flowers offering high quality nutrition (e.g. bees, bumble bees) and it is important for color differentiation (Kring and Schuster 1992; Goldsmith, 1993; Costa and Robb, 1999; Raviv and Antignus, 2004; Döring and Chittka, 2007; Diaz and Fereres, 2007).

Aim of protected cultivation is not only to allow production even under worse climatic conditions (e.g. heavy rainfalls) but to reduce the dependence of frequent pesticide use with all its severe drawbacks (e.g. residues, resistance). However, the use of screens as a physical control has limitations particularly with small insects since too small mesh sizes of nets or complete cover with plastics reduces the efficiency of natural ventilation which is a prerequisite for greenhouses without expensive cooling devices. Materials hindering immigration and being enough air transmissible are desired and UV-blocking materials may be a further milestone in such a development. Therefore, we undertook this study with different combined UV-blocking and UV-transmissible roof and wall materials in small experimental greenhouses to study the immigration pattern of one of the major tomato pest ie, thrips and related virus incidences in the humid tropics to explore the possibilities of designing a chemical free high quality vegetable production system.

MATERIALS AND METHODS

Location

Experiments were conducted on tomato plants (*Lycopersicon esculentum* Mill; family Solanaceae; cv. King Kong II) at the greenhouse complex provided for the AIT-Hanover Project, Asian Institute of Technology, Bangkok, Thailand.

Nets and plastics

Two nets UV-blocking, Bionet® and non UV-blocking (= UV transmitting), Anti Insect® nets (50 mesh: Polysack Plastic Industries, Israel) along with two plastics, UV-blocking (Sun Selector Diffused Antivirus®, Ginegar Plastic Product Ltd**,** Kibbutz, Israel) and UV-transmitting (= non blocking) plastic film, PE-1A (RKW AG, Germany) were used in the experiments (for spectral transmission, Fig. 1).

Treatments and greenhouses

These two nets (UV-blocking and UV non-blocking) and plastics (UV blocking and non-blocking) were permutated in 4 different combinations: UV Blocking nets + UV Blocking Plastics [henceforth referred as B (N+P)]; Non UV-blocking net + UV blocking plastic [NB-N+BP)]; UV blocking nets + UN non-blocking Plastics [BN+N-BP)]; UV Non-blocking nets + UV non-blocking plastic [NB (N+P)]. A total of eight greenhouses (GH) (7.5 m × 2 m × 2 m) were constructed with four GH each placed in identical orientations (either east/west or north/south direction) to avoid any effect of orientation. Furthermore, each greenhouse was provided with two identical doors at the length side. The front and rear end of the door walls were covered with identical nets used for the side walls of each greenhouse. The side-walls of the greenhouses were always covered with either of the nets and the roofs with either of the plastics. Between GH, 1.5 meter space reduced shading from each other. The area around the GH complex was cleaned and all weed plants were removed prior to each series of experiments. Between each series, greenhouses were thoroughly washed and cleaned approximately one week prior to new experiments. A total of three experimental series of five weeks duration were carried out and each experiment was repeated once over the time. A total of 30 potted (25 cm high and 27 cm) tomato plants (2 weeks old) were transplanted in a local media in each greenhouse (pH-5.3; organic matter – 28%; sand – 30%; silt – 39%; clay – 31%; total N – 0.4% ; K – 0.65%; P – 0.18%; Ca – 0.08%). Tomato seedlings were grown in an insect-free evapo-cooled nursery. Radiation triggered and scheduled drip irrigation combined with dosatron fertigation was provided

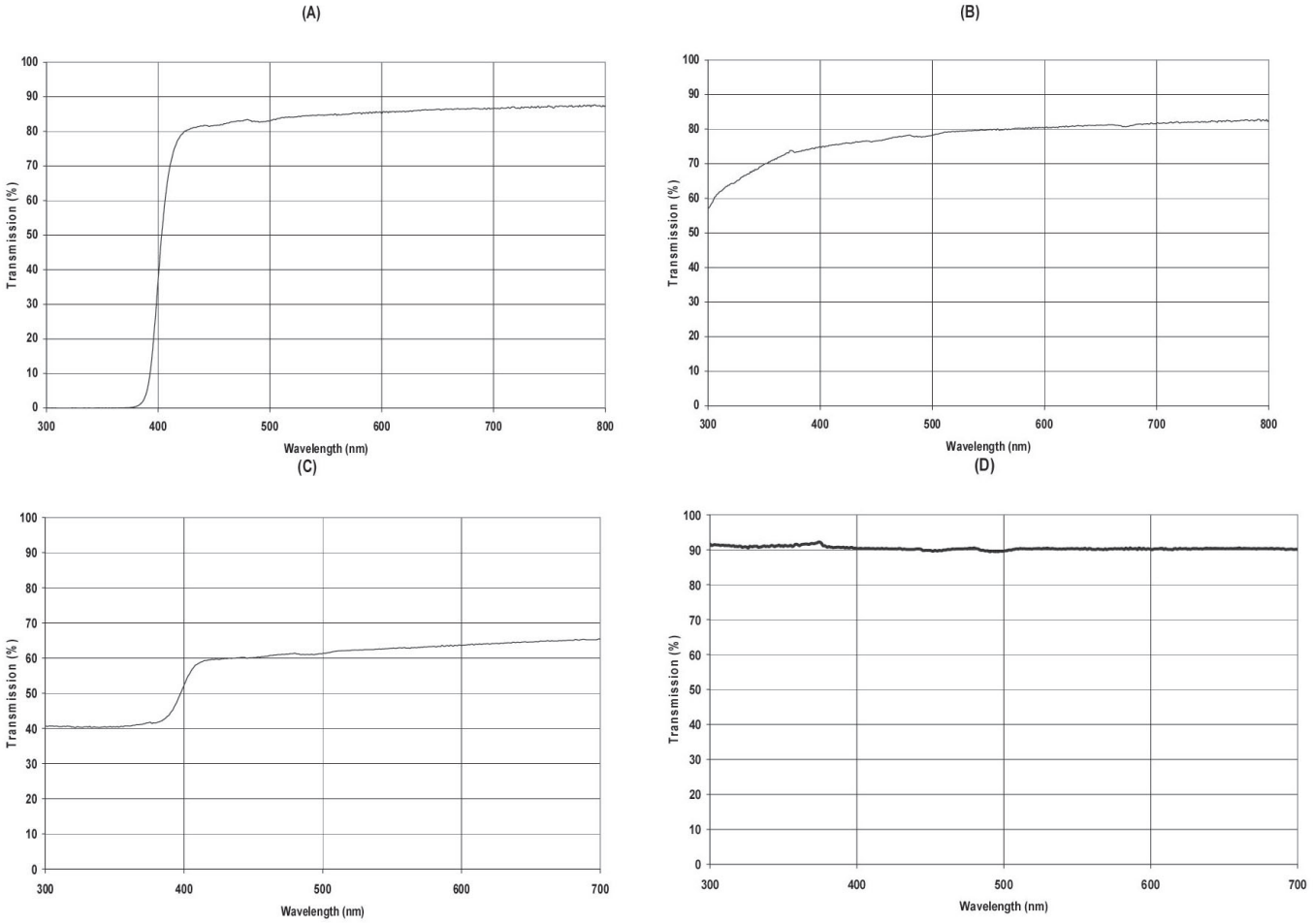

Fig. 1: Spectral transmissivity of UV-blocking plastic film (**A**, Sun Selector Diffused Anti Virus®, Ginegar Plastic Products Ltd., Israel), UV-transmitting plastic, PE-1A (**B**, RKW AG, Germany),UV blocking net (**C**, Bionet®,Polysack, Israel) and UV-transmitting nets (**D**, Anti-Insect®, Polysack, Israel) films measured with a PerkinElmer Lambda 900 UV/VIS/NIR spectrophotometer.

to ensure the mineral balance and optimal growth and development of the tomatoes. Each GH was provided with a temperature, humidity and UV-A using Radiometer UV-Sensor (Dr. Grobel UV-Elektronik GmbH, Germany).

CaCV detection by DAS-ELISA

Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA) was conducted for the confirmation of CaCV-AIT infection of tomato plants (Premachandra, 2004) in addition to symptom diagnostic. Polyclonal and monoclonal antibodies raised against N-protein of Watermelon Silver Mottle Virus (WSMV) and Groundnut Bud Necrosis Virus (GBNV) (Agdia, Inc., Elkhart, ID, USA) were used. Plant leaves were homogenized at a ratio of 1: 5 in PBS-T (2.5 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.14 M NaCl and 0.6 ml/l Tween 20) containing 0.45 polyvinylpyrollidone (PVP). Leaves from healthy plants were used for the control treatment. Absorbance values were read with a microplate reader (BIO-Tek Instruments, Inc, Vermont, USA) at 405 nm, with PBS-T as a blank. The absorbance values were corrected by subtracting the average of three wells of the blank from samples means. Samples having absorbance means three times that of the control was considered as positive. For other viruses e.g. Tomato Yellow Leaf Curl Virus (TYLCV) visual counts were made on the basis of symptoms only.

Exp 1 and 2. Effect of UV blocking nets and plastics on the immigration of thrips and occurrence of tospoviruses (reduced ventilation by partly open doors)

Two rounds of experiment were conducted using above-mentioned set-ups of the 8 GH. The two parallel doors of the GH were simultaneously opened every morning from 6.00-10.00 am coinciding with the peak insect's activities

time (Cohen and Melamed-Madjar, 1978). The immigrating thrips population were measured by blue sticky traps (BST) (25 × 15 cm) positioned half at the plant canopy and half above canopy. The BST were made from Blue PVC sheets coated with insect-glue (Kosfix®, Kosmix Polymer, Bangkok, Thailand) on both sides. A total of 6 BST were placed for each GH, changed two times a week and number of thrips trapped at both side of the traps were counted. Since each trap was considered as one replication total of 10 readings (sampling days) were collected on the immigrating thrips inside each 8 GH during each of the experiments. Additionally, two times a week number of thrips infested leaves were counted from the non-virus infested plants until the fifth week. Two times a week, number of virus infected tomato plants were counted and marked and at the end of the experiments (43 days after transplanting), DAS-ELISA tests were carried out to distinguish between the tospovirus and other viruses e.g. TYLCV. Since the tospovirus was the most commonly occurring one, the plants failed to test positive for the CaCV-AIT infection but showing virus symptoms were assumed to be infected with the TYCLV (referred as other viruses).

Exp. 3 and 4. Effect of UV blocking nets and plastics on the immigration and attraction of thrips and occurrence of tospoviruses (full ventilation with complete open doors)

Two rounds of experiments were carried out in a similar greenhouse set-up as discussed above except that during this round the two doors were kept open during the entire period of experiment. The number of thrips were counted on the BST as per the procedure explained above. Similarly, number of thrips infested leaves and virus infected plants were counted, marked and at the end of experiment, tests were carried out to distinguish between the tospoviruses and other viruses. To investigate the abilities of thrips to reach the experimental GH, two BST each was attached at the outer walls (centrally placed) and traps were changed twice a week followed by counting of thrips. The position and orientation of the traps on all 4 GH types were same. In this way, abilities of the thrips reaching GH were determined.

Statistical Analyses

Adult whiteflies, thrips and aphids on traps, alate aphids and whiteflies on leaves, number of thrips infested leaves, percentage of virus infected plants were subjected to HOVTEST = LEVENE option of SAS (SAS, 1999) to account for homogeneity of variance and normality. In case of non-homogeneity, percent values were transformed using arcsine–square-root (arcsine$\sqrt{}$) transformation. Insects on traps and plants and number of infested leaves count values were transformed by square-root transformation before running an ANOVA followed by mean separation using Fisher's LSD test (Steel and Torrie, 1980; Gomez and Gomez, 1984). Data were then back transformed for presentation as Mean± SE. A significance level of $\mu = 0.05$ was used in for all analysis.

RESULTS AND DISCUSSION

Light Transmission and Temperature

No significant differences in temperatures or humidity inside the four greenhouses were found during either round of experiments. However, the UV light varies under each GH type either during sunny and cloudy days during each four experiments (Fig. 2). The UV levels drops to almost half during cloudy days. During exp. 1 and 2, only 20% of the days were cloudy whereas during experiment 3 and 4 over 40% days were cloudy.

Experiment 1 and 2. Partial Open gates (Partial Ventilation)

Thrips and leaf damage

Thrips, most abundantly recorded species during this experiment and immigration of thrips followed similar trends of entry into UV rich greenhouses over the ones having low UV-intensity. The NB (N+P) GH attracted significantly highest number of thrips over all other greenhouses. Whereas, the B-N + NB-P and NB (N+P) GH types had significantly different immigrating thrips for all sampling dates (Table 1). Even with a different total number

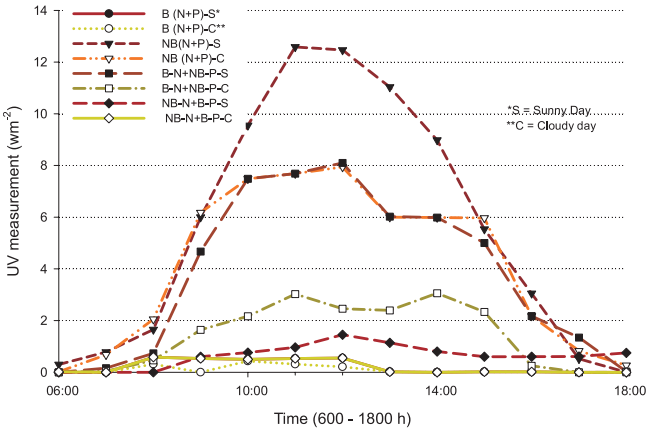

Fig. 2: UV-A measurement (W/m^2) under each four greenhouses, UV-blocking net sidewalls with UV-blocking plastic film as roof [B (N+P)]; UV non-blocking nets as sidewalls and UV non blocking plastic films as roof [NB (N+P)]; UV-blocking nets as side walls and UV non blocking plastic films as roof [B-N+NB-P]; and, UV non blocking nets as side wall and UV-blocking plastics films as roof [NB-N+B=P] using Radiometer UV-Sensor (Dr. Grobel UV-Elektronik GmbH, Germany).

Table 1: Adult thrips trapped on blue sticky traps and cumulative lead damage during experiment 1.

Sampling Dates	Treatments			
	B (N+P)	NB-N+ B-P	B-N+ NB-P	NB (N+P)
Adult/BST				
14-Mar.	0.00±0.00a	0.00±0.00a	0.25±0.13ab	0.33±0.14b
18-Mar.	0.00±0.00a	0.08±0.00a	0.012±0.00a	9.42±0.66b
22-Mar.	0.08±0.08a	0.50±0.19a	3.25±0.65b	8.58±0.96c
25-Mar.	0.17±0.11a	0.17±0.11a	3.50±0.51b	8.83±1.46c
29-Mar.	0.00±0.00a	0.58±0.34a	4.17±0.30b	9.83±1.28c
1-Apr.	0.17±0.11a	0.42±0.19a	7.33±0.70b	23.58±0.36c
5-Apr.	0.17±0.11a	0.67±0.22b	9.92±0.45c	29.75±1.41d
8-Apr.	0.25±0.13a	0.92±0.26a	10.08±0.74b	43.08±4.65c
12-Apr.	0.00±0.00a	1.33±0.41b	11.33±0.53c	80.00±1.69d
15-Apr.	0.00±0.00a	0.42±0.15b	12.75±0.25c	82.67±2.00d
Cumulative leaf damage				
14-Mar.	0.00±0.00a	0.00±0.00a	0.50±0.22b	0.67±0.21b
18-Mar.	0.00±0.00a	0.67±0.21b	1.00±0.45b	2.00±0.26c
22-Mar.	0.17±0.17a	1.00±0.26b	1.67±0.61b	3.17±0.17c
25-Mar.	0.33±0.21a	1.17±0.40ab	2.17±0.70b	5.17±0.31c
29-Mar.	0.50±0.22a	1.33±0.42ab	2.83±0.79b	7.33±0.33c
1-Apr.	0.83±0.31a	1.50±0.50a	3.50±0.72b	9.50±0.34c
5-Apr.	1.17±0.48a	1.67±0.49a	3.83±0.87b	10.33±0.33c
8-Apr.	1.33±0.61a	2.17±0.40ab	4.83±1.17b	12.67±0.33c
12-Apr.	1.50±0.76a	2.67±0.56a	5.83±1.28b	13.17±0.40c
15-Apr.	1.67±0.71a	2.83±0.60a	7.00±1.34b	13.33±0.49c

Means within sampling dates followed by the same letter (s) are not significantly different at P = 0.05. Fisher's LSD test. *Mean + SE

of recorded thrips during second experiment the trends of the immigration remained the same i.e. more in GH with higher UV. The higher number of immigrating thrips inside the NB (N+P) caused significantly high, 52% (13.33 leaves out of average 26 tomato leaves) cumulative damage at the end of 10 sampling days, to the total leaves compared to the 6.59% in the B (N+P) GH. The cumulative percent damage was in the range of 10.90 and 27.31% in the NB-N+B-P and B-N+NB-P GH types (Table 1). The numbers of thrips infested leaves were significantly higher under NB (N+P) GH during second round as well (*data not shown*) indicating preference for thrips to immigrate to the GH with more UV light compared to one having less UV lights.

Virus spread

Cumulative percent incidence of plant viruses was significantly lower with 5.00% in the B (N+P) GH compared to the 40% in NB (N+P) GH types (F = 29.80; df= 3,7; P = 0.0034) at the end 43 day after transplanting (Fig. 3 A). Tospovirus constituted the major proportion out of the total virus and stood at 88 and 66% respectively in B (N+P) and NB (N+P) greenhouse types. Their percent cumulative incidence was too found significant (F = 44.88.78; df= 3, 7; P = 0.0015). In the NB (N+P) GH, the first virus infected plant was recorded 15 days after transplanting (DAT), which spread at faster rates, compared to 27 DAT in the B (N+P) GH and a much slower spread compared to other greenhouses. During the second round of experiments, in total more plants showed virus symptoms and similar to the first round of experiment percent cumulative virus spread was significantly higher under NB (N+P) GH (F = 243.73; df= 3,7; P = 0.0001) at the end of 43 DAT (Fig. 3 B) compared to B (N+P) type GH. However no significant differences were found under B (N+P) and NB-N+B-P types GH. Out of these total, 83.33 % (average 15 plants out of total 18 virus infected plants) were tospovirus. Percent cumulative infestation (or infested plants) with tospovirus were significantly higher under the NB (N+P) type GH (F = 24.30; df= 3, 7; P = 0.005). The virus incidence started earlier at 11 DAT under the NB (N+P) GH types during this round of experiments compared to 31 DAT under B (N+P) GH types. Under the UV blocking plastic GH roof, most of the virus affected plants were found near to the doors, whereas in ones with UV non-blocking roof, infected plant were dispersed all over the GH. The result clearly indicates that the B (N+P) GH type provided the best protection against the tospovirus and probably TYLCV infection over NB (N+P) type GH.

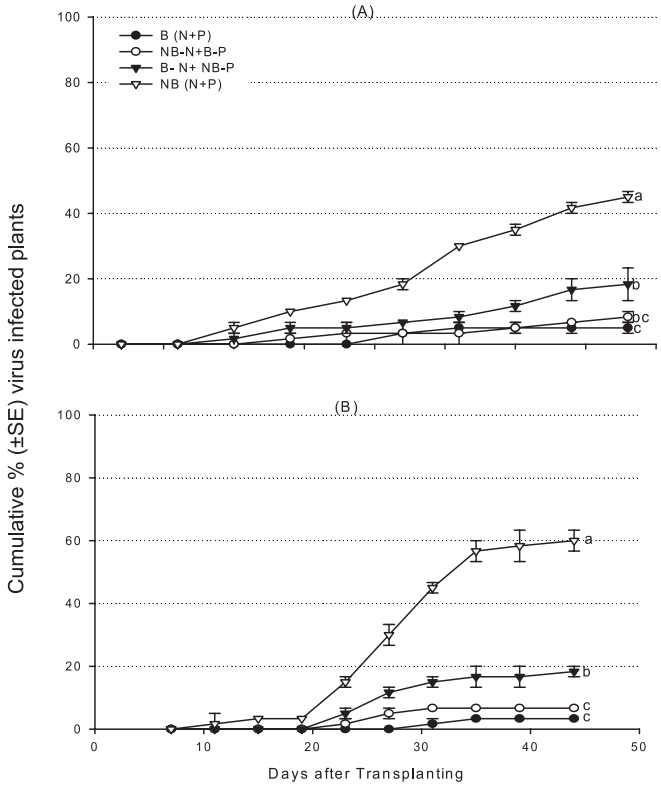

Fig. 3: Percent cumulative virus infected tomato plants under greenhouses, UV-blocking net sidewalls with UV-blocking plastic film as roof [B (N+P)]; UV non-blocking nets as sidewalls and UV non blocking plastic films as roof [NB (N+P)]; UV-blocking nets as side walls and UV non blocking plastic films as roof [B-N+NB-P]; and, UV non blocking nets as side wall and UV-blocking plastics films as roof [NB-N+B-P] during exp. 1 (A) and exp. 2 (B),when greenhouse door open for 600-1000h. Cumulative percent at the 43 days after transplanting sharing a common letter are not significantly different at P <0.05, Fisher's LSD.

Experiment 3 and 4. Open doors (Complete Ventilation)

Thrips and leaf damage

During exp.3, similar to the previously observed trends, significantly higher number of thrips immigrated and captured from the NB (N+P) GH followed by the B-N + NB-P and others. Similar trends of higher leaf damage were observed under NB (N+P) GH. No significant difference was found for most sampling day between B (N+P) and NB-N+B-P type GH (Table 2). Similar trends of thrips immigration and leaf damage with overall lower thrips population were observed during exp.4, a month later.

During experiment, 3, significantly higher numbers of thrips were attracted towards the NB (N+P) type greenhouses for all sampling days followed by B-N+NB-P. However no differences were found among the B (N+P) and NB-N+ B-P type GH (Table 2). Similar significant attraction was found during experiment 4 for the immigrating thrips and a higher number of thrips were attracted towards the GH with high UV intensity.

Virus spread

The high thrips population during exp. 3, resulted into 100% virus infection by 43 DAT, which was significantly higher compared to other GH type (F = 1588.25; df= 3,7; P = 0.0001). The B (N+P) provided significant protection against viruses and only 10% plants found to have virus; 15% under NB-N+B-P and 51% plant under B-N+NB-P GH types (Fig. 4 A.) Out of these total virus infections, over 75% tomato plants tested positive for tospovirus. Tospovirus infection followed the similar trends and lowest infection was recorded under B (N+P) GH type (F = 96.38;

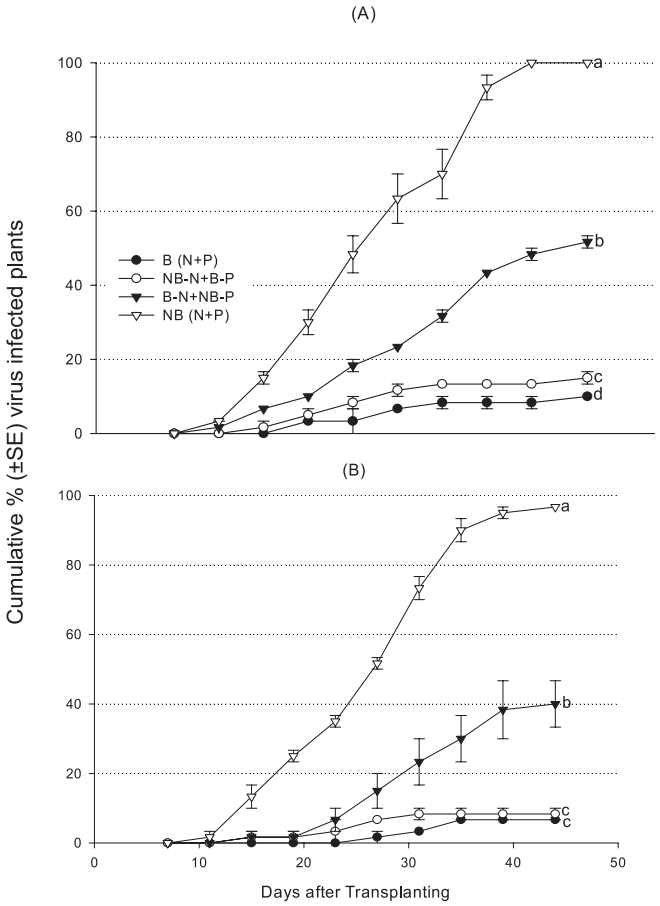

Fig. 4: Percent cumulative virus infected tomato plants under greenhouses (treatments), UV-blocking net sidewalls with UV-blocking plastic film as roof [B (N+P)]; UV non-blocking nets as sidewalls and UV non blocking plastic films as roof [NB (N+P)]; UV-blocking nets as side walls and UV non blocking plastic films as roof [B-N+NB-P]; and, UV non blocking nets as side wall and UV-blocking plastics films as roof [NB-N+B-P] during exp. 3 (A) and exp. 4 (B), when greenhouse doors kept open (complete ventilation). Cumulative percent at the 43 days after transplanting sharing a common letter are not significantly different at P <0.05, Fisher's LSD.

Table 2: Thrips trapped on blue sticky traps and captured outside walls of the greenhouses and cumulative leaf damage during experiment 3.

SamplingDates	Treatments*			
	B (N+P)	NB-N+ B-P	B-N+ NB-P	NB (N+P)
Adult/BST				
10-Jun	1.00±0.17a	1.25±0.28a	0.83±0.27a	13.00±5.24b
14-Jun	2.33±0.74a	3.00±0.83a	16.00±1.71b	47.50±7.43c
17-Jun	1.67±0.51	2.42±0.57	27.75±4.08	77.17±12.82
21-Jun	2.50±0.90a	3.50±1.22a	34.08±18.79b	193.25±37.05c
24-Jun	5.75±1.61a	9.25±1.74a	48.83±4.41b	166.75±20.41c
28-Jun	5.67±1.94a	7.83±1.96a	56.75±11.82b	145.83±32.90c
1-Jul	7.92±2.33a	10.08±3.00ab	49.92±16.64b	209.50±32.52c
4-Jul	8.42±2.52a	17.25±2.18a	52.08±10.24b	232.67±25.24c
7-Jul	7.17±1.60a	10.08±3.00a	45.92±12.29b	270.50±30.79c
11-Jul	4.58±1.40a	14.67±2.35a	54.25±17.46b	293.33±26.51c
Cumulative leaf damage				
10-Jun	0.33±0.21a	0.67±0.21a	1.50±0.22b	2.67±0.21b
17-Jun	1.33±0.33a	2.00±0.00b	4.17±0.31c	8.67±0.42d
24-Jun	1.67±0.42a	2.67±0.21b	5.83±0.40c	11.17±0.48d
1-Jul	1.83±0.48a	3.67±0.21b	8.17±0.40c	14.00±0.63d
11-Jul	2.33±0.33a	5.00±0.37b	11.33±0.33c	21.00±0.68d
Thrips/BST outside wall				
10-Jun	0.63±0.26a	0.75±0.25a	2.63±0.18b	5.63±0.50c
14-Jun	1.50±0.50a	1.75±0.25a	4.13±0.40b	14.25±1.53c
17-Jun	1.13±0.23a	1.63±0.26a	9.50±1.35b	21.50±1.76c
21-Jun	1.13±0.30a	1.63±0.18a	10.13±0.97b	36.13±2.87c
24-Jun	1.38±0.26a	1.88±0.13	14.63±0.82b	51.63±7.80c
28-Jun	1.88±0.30a	2.13±0.30a	18.38±1.05b	69.25±5.30c
1-Jul	2.13±0.30a	2.63±0.18a	20.50±2.20b	65.13±7.05c
4-Jul	2.63±0.42a	3.25±0.31a	18.75±1.37b	70.25±5.95c
7-Jul	2.13±0.44a	3.13±0.44a	16.38±0.89b	68.38±5.86c
11-Jul	1.36±1.40a	1.67±1.73a	11.22±11.68b	77.50±6.65c

Means within sampling dates followed by the same letter (s) are not significantly different at P = 0.05. Fisher's LSD test.
*Means + SE

df= 3, 7; P = 0.0003). First virus symptoms appeared under NB (N+P) GH at 11 DAT, whereas for the B (N+P), it only appeared at a week later on 19 DAT and spread at slower rate compared to the plants grown under NB-P roofs GH types. The virus spread followed similar trends during exp.4, where albeit less overall cumulative infection at 96% under NB (N+P) was recorded at 43 DAT, which was significantly higher to other GH types (F = 196.94; df= 3,7; P = 0.0001). However, no significant difference found among B (N+P) and NB-N+B-P GH types (Fig. 4 B) and once again the GH with B (N+P) provided highest level of protection against virus. Similarly, the incidence of tospovirus was significantly different in 4 GH types, except no difference found under plants grown in the two GH types with UV-blocking plastic roofs. Similar to the virus spread under the exp. 1 & 2, under the UV blocking plastic GH roof, most of the virus affected plants were found near to the door, whereas in ones with UV non-blocking roof, infected plant were dispersed all over the GH. The results clearly indicate the better protection provided by the GH made from the B (N+P) over others not only in terms of reduction of total virus plants but also the delayed infection.

Thrips immigration and leaf damage

Thrips, *Ceratothripoides claratris*, gave a very sensitive response to the changes in UV-environment and irrespective of ventilation period (partial or complete), preferred to immigrate inside UV-rich environment in a concentration-dependent manner. During exp. 1 and 2, limited hours of open doors (limited ventilation), a total of 0.83 and 5.42 thrips were trapped over 384 and 382 under B (N+P) and NB (N+P) GH types respectively. Moreover, when gates were kept open (full ventilation) during experiment 3 and 4; a higher total of 48 and 50 and 1664 and 1070 thrips on BST were trapped under B (N+P) and NB (N+P) GH types respectively. When incoming radiation were measured inside these structures (Fig. 2), roofs of the houses were more important over side nets that determines the amount of UV inside a GH and wherever, the UV-blocking plastics were used as roof, the incoming UV-radiation were

considerably less. Consequently, the immigrating thrips showed an UV-concentration-dependent behaviour. For instance, during experiment 1, on a typical sunny day at 1200 h, GH types NB (N+P) recorded higher UV 12.47 w/m^2 followed by 8.10 w/m^2 in the B-N+NB-P, 1.45 w/m^2 under NB-N+B-P and 0.55 w.m2 under B (N+P) type GH (Fig. 2). These UV radiations reached to their half level in respective GH types during cloudy days without any major changes in the immigration and plant colonisation (see below the leaf damage part). This indicates that it is not the absolute UV amount available triggers thrips selection behaviour but the relative difference between two light environments plays important role in determining the flight and immigration behaviour of thrips.

Higher immigrating thrips inside NB (N+P) type GH resulted into higher percentages of the leaf damage, the cumulative percentage of leaf damage at the end of 43 DAT under NB (N+P) type GH was 51.59 and 56.15 against 6.59 and 7.26 under B (N+P) GH type during experiment 1 and 2 respectively. With the increase in total number of thrips during experiment 3 and 4, higher percent cumulative leaf damage was recorded. Under NB (N+P) the damage was 81.28 and 71 % against 9.18 and 11.16% during experiment 3 and 4 respectively.

The results are in consistent with the previously reported work on WFT, *Frankliniella occidentalis* (Pergrande) from Israel or other thrips species, where significant reduction of the thrips were reported under UV-absorbing plastic tunnels (Antignus *et al*., 1996b; Kumar and Poehling, 2006). Similarly, in a choice study of Costa *et al*. (1999) captured 90-98% of released thrips, WFT, *Frankliniella occidentalis* (Pergrande) under tunnels rich in UV over tunnels covered with UV-absorbing plastics. The results are in disagreement with the previously reported work of Antignus *et al*. (1998) where no difference in immigration preference of another thrips species, *Frankliniella occidentalis* was found under 50-mesh Bionets. The difference in these two results could be because of different set-ups, species of the thrips and use of a combination of UV-blocking plastics and nets in compare to only nets. Similarly, the results on leaf damage are in agreement with studies with other species of thrips like Vos *et al*. (1995), reported reduced leaf damage by thrips on peppers planted on silvery plastic mulch compared with control. This could be due to the fact that reflectance pattern of UV wavelengths is important in determining whether thrips, *F. occidentalis* alight on a host. The ability of thrips to receive light in the UV range spectrum is well documented (Matteson *et al*., 1992) even a differentiation between UV-A and UB-B. Mazza *et al*. (1996, 2002) showed that the thrips *Caliothrips phaseoli* avoids UV-B but is attracted by UV-A and Vernon and Gillespie (1990) reported that high UV reflectance environment repels thrips. The selective sensitivity of thrips to different UV ranges becomes obvious when we compare our results with reports on the use of UV-reflective mulches against thrips.

Plant Virus

Thrips, *Ceratothripoides claratris* is recently reported to be serious pests of protected cultivation tomato crops form greater Bangkok area and vector of tospovirus, CaCV (isolate AIT) (Premachandra, 2004).

Number of plants showing virus symptoms, which was later confirmed through ELISA test, followed the trends of the immigrating thrips and WF, which was recorded least under the B(N+P) type GH over NB (N+P) type GH. B (N+P) type GH reduced and delayed the virus infection in all experiments. Majority of virus were the tospovirus as evident through the thrips as most occurring species. However, no further attempts were made to isolate other viruses but presumed that they were Tomtao Yellow Leaf Curl Virus (TYLCV). In Israel, the spread of TYLCV were significantly reduced using UV-absorbing nets (Antignus, *et al*., 1998). Similarly, a highly significant reduction of 80% (UV-absorbing plastic) over 1% under control (non-UV absorbing plastics) of TYLCV incidence was reported (Antignus *et al*., 1996a; Gonzalez, 2004). The incidence of *cucurbit yellow stunting disorder virus* in melons were reported to be 70% less under UV-absorbing films and the same film appeared to be effective against aphid-borne *Zucchini yellow mosaic virus* (Antignus, 2000).

In conclusion, our result shows that the greenhouses made from combination of the UV-blocking nets as side walls and roof with UV- blocking plastics are able to deter the immigrating thrips and consequently tomato plants grown under such GH had fewer leaf damage and eventually decreased virus infection including those of tosposvirus. Being in the tropics, the major amount of light filters through the roof, having UV-blocking plastic on roof, significantly reduced the incoming UV into the structure, leaving the limited UV-filtration task for the side walls. At the same time, overall little differences were found among the GH with the non UV-blocking net sidewalls and UV-blocking roof in terms of immigration of insects, leaf damage and virus spread. Thus, such greenhouses present itself as a viable option over all plastic greenhouses in the humid tropics.

Reducing immigration of the pests in greenhouse leads to a lower initial pest population density, which is a key factor for successful and effective control in general (Xu *et al*., 1984). Other potential benefits from the reduced UV-environment achieved through the use of UV-blocking net and plastics may include improved performance of

entomopathogenic fungi (Costa *et al*., 2001) and baculoviruses (Goulsom *et al*., 2003), improved management of some fungal pathogens (Reuveni and Raviv, 1992, 1997; Elad, 1997), reduced UV related degradation of botanicals like neem (Johnson *et al*., 2003; Barrek *et al*., 2004) and overall improvements in the microclimate leading to healthier production of crops like tomatoes.

Recently most publications reported that under climate change scenario the sucking pest and plant viruses will increase so does the plant diseases (Canto and Aranda, 2009). Clearly a low UV environment facilitated through optimal combination of low-cost nets and plastics could provide a sustainable environment for safer and non-pesticide produce, which would be a much needed and sought after by consumers and exporters. However, it has been also reported that reduced UV could have some negative effects on some plant species. It is suggested that more studies should be carried out using different crops and pest models to further refine the idea into working technologies. Similarly, in the case where release of parasitoids and predators are carried out, being arthropods, further studies are also suggested.

Finally, the increased surge in number of plant viruses due to sucking pest, and resulting losses to the yield and quality of vegetable crops, could be reduced directly by adopting to the low UV growing condition under various plasticulture production system that would require minimal use of chemical pesticides for sustainable pest management in the context of climate change.

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Diversity analysis of *Alternaria porri* (Ellis) Cif - causal organism of purple leaf blotch of onion

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ABSTRACT

Purple leaf blotch (PLB), caused by *Alternaria porri* (Ellis) Cif, is the most destructive disease of onion in India. Phenotypic and genetic diversity of 19 *A. porri* isolates collected from different farms in Karnataka state of India was characterized based on morphology, aggressiveness, response to the fungicides (chlorothalonil and mancozeb), and genetic analysis using sequencing of ITS region of rDNA. All *A. porri* isolates produced typical orange color pigmentation on potato dextrose agar (PDA). Isolates exhibited significant differences in their aggressiveness on onion variety, Arka kalyan and could be categorized into highly aggressive, moderately aggressive and less aggressive groups. Comparative fungicidal assays indicated that isolates were more sensitive to chlorothalonil than to mancozeb. EC_{50} values for inhibition of mycelia growth ranged from 3.07 to 48.30 µg/ml for chlorothalonil and 33.22 to 76.90 µg/ml for mancozeb. Isolates could be separated into two groups based on phylogenetic analysis of ITS sequences; Clade I contains highly and moderately aggressive isolates and clade II contains less aggressive and nonpathogenic isolates. Thus, the present study demonstrated existence of phenotypic and genotypic diversity among *A. porri* isolates collected from areas of commercial onion production, which has implications in breeding onion for resistance to purple blotch.

Key words: Onion, purple leaf blotch, *Alternaria porri*, onion, genotypic diversity

INTRODUCTION

Onion is considered as one of the five most important fresh market vegetable crops (Cramer, 2000). Onions are used both as food and seasoning, the immature and mature bulbs are eaten raw or they may be cooked and eaten as a vegetable (Messiaen, 1994). Onion contains the lachrymatory agent, a strong antibiotic, with fungicidal, bacterial and nematicidal properties (Purseglove, 1972). The world production of onion including shallots was 3.56 lakh tonnes in 2.8 lakh hectares (FAO, 2008). India ranks second in area and third in production with 6.66 million tonnes in 0.478 million hectare area. Onion is susceptible to various foliar, bulb and root pathogens that reduce yield and quality (Cramer, 2000). Among these, purple leaf blotch (PLB) caused by *Alternaria porri* (Ellis) Cif, is one of the most important fungal diseases of onion (*Allium cepa* L) affecting both bulb and seed crop (Awad *et al*., 1978; Everts and Lacy, 1990a; Brar *et al.*, 1990; Aveling *et al*., 1993, 1994 and Cramer, 2000; Dhiman and Chada, 1986), causing losses up to 100 per cent (Singh *et al.*, 1992). The disease is characterized by the development of sunken purple necrotic lesions on the foliage and seed stock that progressively coalesce, leading to premature drying of leaves and cessation of bulb development (Gupta *et al.*, 1994).

Under the favorable conditions of warm temperatures and prolonged leaf wetness, onion foliage may rapidly succumb to blight (Everts and Lacy, 1996; Suheri and Price, 2001). Premature loss or collapse of foliage may result in significant yield reductions in onion fields. To maintain crop healthy, the onion growers use integrated strategies for management of PLB, including the use of pathogen free seed, crop rotation, planting of disease resistant varieties, disease forecasting models, and most notably, the application of protectant fungicides, chlorothalonil and mancozeb (O'Higgins, 1988; Gupta *et al*., 1996; Gupta and Pathak, 1988; Srivastava *et al.*,1996; Sharma, 1987). Artificial inoculation experiments in controlled environments and screening under natural epiphytotic led to development of moderate level of resistance in onion germplasm for resistance to PLB (Sharma, 1997; Sugha *et al*.,1992; Dhiman and Chada, 1986). Pathogen diversity plays significant role in determining the variation in response of host and fungicides to PLB. However, the existence of pathogen diversity with regard to aggressiveness on host and fungicidal sensitivity has not been elucidated. There is concern that resistant individuals within a fungal population may develop due to repeated application of these fungicides. There are several examples of pathogen populations that express reduced sensitivity to fungicides under the selection pressure from repeated

fungicide use (Eckert,1988). For example, Holm *et al.* (2003) reported considerable variability in *A. solani* isolates to chlorothalonil and mancozeb due to repeated exposure. Monitoring *A. porri* isolates is necessary to better understand population responses to these compounds and the potential for continued control of PLB.

Variation in phenotypic, genotypic and biochemical traits within populations of *Alternaria* spp., has been reported (Aradhya and Chan, 2001, Naresh and Sangwan,. 2003, Remneva and Ivanyuk, 1968; Rotem, 1966; Van der Waals *et al.*, 2004; Weir, *et al*., 1998; Kumar *et al*., 2008; Rogers and Stevenson, 2010). Morphological and pathogenic variability among isolates of *A. solani* has given rise to claims of the existence of races (Rotem, 1966). Molecular analysis including nucleotide sequence of the internal transcribed spacer (ITS) regions of rDNA is a useful tool for studying phylogenetic relationships of filamentous fungi and for relating it to host-specificity, geographical variation, and gene-diversity among intra- and interspecies groups (O'Donnell, 1993). To date, no research has determined diversity in aggressiveness in populations of *A. porri* in relation to phenotypic and genotypic characteristics. The objectives of this study were to examine morphological, molecular, the aggressiveness and *in vitro* sensitivity of *A. porri* to commonly used fungicides to control PLB and to correlate phenotypic characters with genotypic markers.

MATERIALS AND METHODS

Collection, isolation and maintenance of isolates

Onion leaves showing typical symptoms of PLB were collected from different onion farms located in Karnataka during July-October crop season of 2009 and 2010. To obtain isolates, a small piece of tissue (5 × 5mm) was taken from the margin of the lesion, surface sterilized in 1% sodium hypochlorite for 3 min, rinsed with sterile water, and placed on potato dextrose agar (PDA). The plates were incubated under cool white fluorescent lights at 25 °C ±1 °C for 7days. Then, the plates were examined with a dissecting microscope (×15 magnification) and a small agar plug with a single germinating spore was picked from each plate with the use of a sterile surgical blade and transferred to a new PDA plate. A total of 19 *Alternaria porri* isolates were collected (Table.1). The cultures were maintained on PDA slopes under liquid paraffin oil at 8 °C.

Morphological examination

For colony morphology, five mm diameter disks cut from colonies derived from single germinated conidia were transferred to Petri dishes (80mm) containing PDA and incubated at 25 °C ±1 °C for 10 days (Pryor and Michailides, 2002). After incubation, cultures were examined for colony color, colony margin, colony texture, and the development of pigments in the agar medium (Pryor and Michailides, 2002). For sporulation, five mm diameter disks were transferred to Petri dishes containing 0.05 × PDA. Dishes were incubated under continuous light at 25 °C ±1 °C for 7 days. After incubation, cultures were examined for sporulation.

Aggressiveness

The aggressiveness of the isolates was determined by inoculating detached onion leaves according to the method of Pryor and Michailides (2002) with slight modifications. Fully matured third leaf from bottom were obtained from 60 days old plants during July-October crop seasons of 2010 and 2011. The leaves were surface sterilized by immersing them in a 1% sodium hypochlorite solution for 1 min before inoculation, then rinsed three times in sterile distilled water and subsequently air dried. The middle portion of the leaves were then pinpricked and placed on wire mesh platforms in plastic boxes (23 × 31 × 10 cm, length by width by height). Inoculation was carried out on the wounded leaves by placing 20 µl of a 2.5 x 10^5/ml H_2O conidial suspension on each wound. Leaves used as control were inoculated with 20 µl of sterile distilled water. Sterile distilled water was added in each box and then covered to maintain high relative humidity. The containers with the inoculated leaves were incubated at 25 °C ±1 °C for 7 days. The infection was recorded by measuring the length and width of the lesions on each leaf seven days after the inoculation. Since the lesions are elliptical in shape, the lesion area was calculated using the formula µ × l × w, where the l= half length of lesion and w= half width of lesion (Fagan, 1988).

Fungicide sensitivity determination

The commercial formulations of mancozeb (80%WP) and chlorothalonil (75%WP) were used in the present study. Sensitivity of *A. porri* isolates to mancozeb and chlorothalonil were evaluated using mycelia growth assays. Mycelial disks (5 mm in diameter) of the fungal isolates, removed from the margins of 7-day-old culture, were inoculated to PDA amended with mancozeb and chlorothalonil at concentrations of 0, 0.1, 1.0, 10.0, 100 and 1000 µg/ml. Petri plates without the fungicide were used as controls. Three replicates per concentration were used for each isolate. The radial growth (colony diameter) of each isolate was measured in two perpendicular directions, with the original mycelial plug diameter (5 mm) subtracted from this measurement, after 7 days of incubation at 25°C ±1°C in the dark. The percent growth inhibition for each isolate and fungicide was determined by using the formula suggested by Vincent (1947). EC_{50} values were calculated for each isolate and each fungicide by regression

Table 1: *Alternaria porri* isolates used in this study

Isolate No	Year of isolation	Location	Gene bank accession No	Genetic group
OOA2	2009	Hessaraghatta	JF710495	I
OOA3	2009	Doddaballapur	JF710499	I
OOA5	2009	Gouribidnur	JF710486	I
OOA6	2009	Malur	JF710488	I
OOA8	2009	Sira	JF710494	I
OOA11	2009	Challekere	JF710496	I
OOA12	2009	Chitraduraga	JF710497	I
OOA13	2009	Rannebenur	JF710491	I
OOA14	2009	Haveri	JF710489	I
OOA15	2010	Dharawad	JF710485	I
OOA17	2010	Kolar	JF710483	I
OOA19	2010	Chintamani	JF710490	I
OOA20	2010	Hiriyur	JF710487	II
OOA21	2010	Gubbi	JF710481	II
OOA22	2010	Arsikeri	JF710493	I
OOA23	2010	Hassan	—	I
OOA24	2010	Bagalakota	—	I
OOA25	2010	Hosakote	—	I
OOA29	2010	Birur	JF710498	I

analysis of the percent inhibition of fungal growth versus the $\log_{10}$ of the fungicide concentration (µg/ml). Analyses of variation of EC_{50} values from three experiments were conducted using SAS Proc ANOVA followed by mean separation using Fisher's LSD in SAS (SAS Institute).

Molecular characterization

DNA extraction: Total fungal DNA was extracted from mycelia obtained from 100 ml of potato dextrose broth (PDB) for 7 days at 25 ±1 °C. Mycelium was harvested from liquid cultures by filtration through Whatman no.3 filter paper and damp dried and subsequently ground into a fine powder in liquid nitrogen. DNA was extracted from the frozen mycelial powder employing a slightly modified method of Raeder and Broda (1985) by incubating at 37 °C for 10 min after the phenol: chloroform: isoamyl alcohol (25:24:1) precipitation. This was followed by precipitation with 0.54 volumes of isopropyl alcohol and centrifugation at 10,000 g for 2 min. The DNA pellet was washed with 70% cold ethanol, dried at room temperature overnight (16hr) and then pellet was re-suspended in 30 µl 10mM TE buffer (pH.8) DNA was stored at -20 °C.

PCR amplification: ITS region of r DNA of fungal isolates were amplified by PCR with universal primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3' (White *et al*., 1990). PCR reactions (50 µl) were performed with primer pair ITS1/ITS4 in Eppendorf master cycler by 34 cycles of denaturation at 94°C for 60s, annealing at 55 °C for 60s, and extension at 72 °C for 1.5 min with an initial denaturation of 5 min at 94 °C before cycling and final extension of 5 min at 72 °C after cycling. Amplified PCR products were separated in 2 % agarose gels in Tris- Borate-EDTA (TBE) buffer and visualized under UV after staining with ethidium bromide (0.5µg/ml).

Sequencing of the ribosomal ITS region: Amplified ITS region of r DNA products (560bp) with primer pair (ITS1 and ITS4) were purified using PCR product purification kit KT 72 (Genei, Bangalore). The purified product (10-12 ng) was used for PCR cycle sequencing using Big Dye Terminator ready reaction Mixture kit (Applied Bio systems, USA) and analyzed with ABI 3100 analyzer capillary machines. The sequences were deposited in the Gene Bank (www.ncbi.nlm.nih.gov) and the accession numbers are given in the Table 1.

Phylogenetic analysis: The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.06225136 is shown (Fig. 2). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, *et al*., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 690 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura, *et al*., 2007).

RESULTS AND DISCUSSION

Morphological characters

Isolates produced colonies were either greyish orange or brownish orange in color. Colonies generally had a cottony texture, the reverse side of the colonies were yellowish or dark orange. Majority of the isolates produced dark orange pigment while two isolates (OOA2 and OOA29) produced yellowish orange pigment on PDA after seven days of inoculation. Most of the cultures exhibited cottony colonies with circular margin and isolates OOA2, OOA3, OOA25 and OOA250 had smooth colonies with circular margin (Table.2). The isolates significantly differed in seven days of radial growth. The isolates were classified as three groups based on radial growth; fast (46.0-53.6mm, OOA13,OOA14, OOA19, OOA21 and OOA24), intermediate (40-45mm, OOA2, OOA8, OOA11, OOA15, OOA20, OOA23 and OOA29) and slow (28.66-39mm, OOA3, OOA4, OOA5, OOA6 and OOA22) (Table.3). Sporulation was not recorded on half strength PDA after seven days of inoculation and scanty conidiogenous hyphae (Chlamydospore like structure) were present.

Aggressiveness

The isolates of *A.porri* differed significantly ($p < 0.05$) in aggressiveness (Table 3**).** Among 19 isolates of *A. porri*, OOA24 was recorded as highly aggressive, OOA21 as less aggressive and OOA20 as non aggresssive. While remaining isolates were rated as moderately aggressive.

Fungicidal sensitivity

The isolates exhibited significant differences ($p<0.05$) in sensitivity to mancozeb and chlorothalonil in EC_{50} values (Table 3). When tested against chlorothalonil, EC_{50} values ranged from 3.07 to 48.30 µg/ml, and the mean value across the population was 22.90 µg/ml. One isolate (OOA17) of the 19 isolates was highly sensitive (EC_{50} 3.07 µg/ml). Population responses to mancozeb were substantially lower than chlorothalonil (Table 3). Mean EC_{50} values ranged from 33.22 to 76.90 µg/ml with a population mean of 51.69 µg/ml.

Molecular characterization

The DNA from all 19 isolates of *A.porri* yielded single PCR product of 560bp when amplified with ITS1 and ITS4 (Fig.1). In a neighbor-joining tree constructed from the ITS and 5.8S rDNA sequences, *A.porri* isolates fell into two clades (Fig.2). Clade I contains moderately and highly aggressive isolates and clade II contains less aggressive and non-pathogenic isolates.

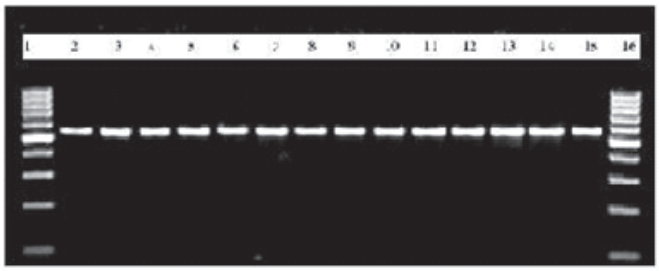

Fig. 1: PCR amplification of ITS region of r DNA of A.porri isolates

Table 2: Colony morphologies of *Alternaria porri*

Isolate No	Colony colour	Colony texture	Zonation	Pigmentation
OOA2	Greyish orange	Felty	Concentric	Yellowish orange
OOA3	Greyish orange	Felty	No	Dark orange
OOA5	Brownish orange	Wolly	No	Dark orange
OOA6	Brownish orange	Wolly	No	Dark orange
OOA8	Brownish orange	Wolly	No	Dark orange
OOA11	Brownish orange	Wolly	No	Dark orange
OOA12	Brownish orange	Wolly	Concentric	Dark orange
OOA13	Brownish orange	Wolly	Concentric	Dark orange
OOA14	Brownish orange	Wolly	No	Dark orange
OOA15	Brownish orange	Wolly	No	Dark orange
OOA17	Brownish orange	Wolly	No	Dark orange
OOA19	Brownish orange	Wolly	No	Dark orange
OOA20	Brownish orange	Wolly	Concentric	Dark orange
OOA21	Brownish orange	Wolly	Concentric	Dark orange
OOA22	Brownish orange	Wolly	No	Dark orange
OOA23	Brownish orange	Wolly	No	Dark orange
OOA24	Greyish orange	Wolly	Concentric	Dark orange
OOA25	Greyish orange	Felty	No	Dark orange
OOA29	Greyish orange	Felty	No	Yellowish orange

Table 3: Colony growth, aggressiveness and fungicidal sensitivity of *Alternaria porri* isolates

Isolates	Growth rate(mm)[1]	Aggressiveness (mm^2)[2]	EC_{50} Values	
			Chlorothalonil	Mancozeb
OOA2	52.0±1.7	327.2±50.56	34.2	44.1
OOA3	32.0±5.2	279.5±65.7	13.7	57.6
OOA5	38.0±0.0	315.5±45.42	30.9	76.9
OOA6	28.6±4.0	319.2±89.56	13.7	43.7
OOA8	43.3±1.5	296.6±59.05	15.0	48.9
OOA11	41.6±4.9	276.9±63.33	20.9	72.5
OOA12	36.3±4.7	155.0±50.4	15.0	59.4
OOA13	48.3±1.5	248.6±57.0	21.5	43.5
OOA14	46.0±1.7	316.25±39.4	48.3	39.9
OOA15	40.0±2.0	248.2±68.3	10.6	68.8
OOA17	33.33±1.1	283.7±83.2	3.07	33.2
OOA19	53.33±1.5	187.2±48.7	23.2	47.6
OOA20	43.3±4.1	0	17.8	62.2
OOA21	53.6±2.52	122.4±54.97	31.4	36.3
OOA22	39.0±6.5	297.6±69.8	11.0	51.6
OOA23	40.0±5.0	267.7±66.0	26.7	40.5
OOA24	52.0±3.4	384.3±58.4	35.9	49.6
OOA25	34.6±1.53	197.0±54.57	24.3	47.5
OOA29	40.0±0.0	227.0±44.8	17.8	60.7
CD5%	8.11	101.89		

[1]Mean of three replications± standard deviation. Each replication consists of 9 Petri plates [2]Mean of three replications± standard deviation. Each replication consists of 12 detached leaf lets

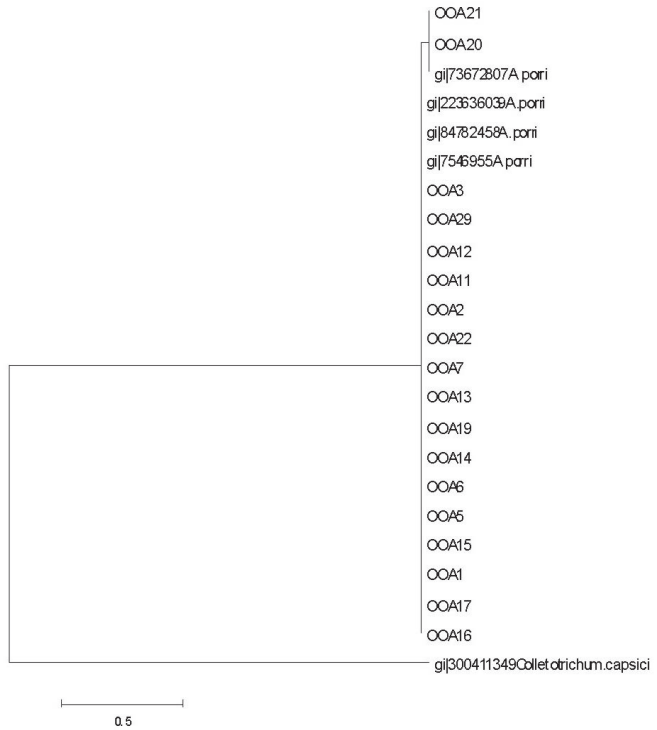

Fig. 2: Internal transcribed spacer (ITS) and 5.8S sequence-based tree generated using neighbor-joining analysis

Development of resistance and reduced sensitivity to fungicides and increased aggressiveness within populations of *Alternaria* spp are emerging as serious problems for disease management (Holm *et al*., 2003; Naresh and Sangwan, 2003; Gupta *et al*., 2004: Pasche *et al*., 2004; Thrall *et al*., 2005; Rogers and Stevenson, 2010). For effective management of PLB of onion, a clear understanding of fungicide sensitivity and aggressiveness in populations of *A. porri* derived from different crop backgrounds is required. We assumed that change in cropping patterns, introduction of new crop varieties and repeated applications of protectant fungicides may cause change in structure of *A. porri* populations. In present study, an attempt was made to identify and characterize variability among isolates of *A. porri* obtained from different geographic regions of onion production in Karnataka state using biologically relevant phenotypic criteria such as aggressiveness and *in vitro* fungicidal response and genotypic printing using nucleotide sequences of ITS region of rDNA. Results obtained in this study showed that there were differences in the degree of sensitivity to mancozeb and chlorothalonil within isolates of *A. porri*. These findings are in agreement with results obtained for *A. solani*, where distinctions in sensitivity to mancozeb and chlorothalonil were apparent (Holm *et al.*, 2003). Similarly, differences in sensitivity to azoxystrobin, boscalid and chlorothalonil within populations of *A. dauci* have been reported (Rogers

and Stevenson, 2010). These studies indicates that diversity in isolate sensitivity to fungicides is evident in *Alternaria* spp. and this is possible due to selection pressure from repeated fungicide applications (Eckert,1988). Despite differences in sensitivity to mancozeb and chlorothalonil within populations of *A. porri*, the response to fungicides appears within the range expected for control. Resistance build up is not apparent within populations of *A. porri* to both fungicides used in the present study due to their multi site inhibiting mechanisms and development of resistance to these class of fungicides is very low (Lorenz, 1988). However, decreased sensitivity to mancozeb and chlorothalonil among USA populations of *A. solani* (Holm *et al*., 2003) and Mexican populations of *Phytophthora infestans* (Sujkowski *et al*., 1995) have been recorded. Isolates of *A. porri* in the present start also showed variation in sensitivity to the fungicides and the EC_{50} values revealed that chlorothalonil was more effective than mancozeb.

Alternative to chemical control, the use of resistant onion cultivars is an efficient method of PLB management. Different sources of resistance to *A. porri* have been reported in onion. Among the genotypes and varieties that exhibit resistance to PLB are IC48059, IC48179, IC48025, ALR PBR 322, 324, 302, 310, 298, 287 Agrifound Light Red, Agrifound Dark Red, Red Glo VLPiyaz (Sugha *et al*., 1992; Sharma, 1997; Mani *et al*., 1999). However, as with fungicides, resistance can be overcome by the development of new pathogenic genotypes in *A. porri*. Therefore, it is necessary to search for a sustainable method for disease management, which necessitates a better understanding of the genetic variability in populations of *A. porri*. By understanding population genetic structure, adequate decisions can be made about disease management with fungicides and breeding programs. The pathological assays indicated that most of the isolates of *A.porri* are highly aggressive and few are less aggressive or non-pathogenic on Arka kalyan. Similar results have been reported for *A. brassicicola*, where distinctions in isolate aggressiveness were apparent on *Cakile maritima* (Thrall *et al*., 2005). Rotem (1966) reported existence of pathotypes with varying aggressiveness within *A. solani* on potato. In addition, variability in aggressiveness within populations of *A. brassicae* on mustard have been recorded (Gupta *et al*., 2004; Naresh and Sangwan, 2003).

Virulence and aggressiveness testing using a set of host differentials and fungicidal sensitivity have been used to identify markers for genetic variation studies. These techniques are labor-intensive, time-consuming and often generates variable results that are influenced by the environment and inoculation techniques. The analysis of sequences of ITS and β-tubulin and random amplified polymorphic DNA have been used as genetic markers for differentiation of fungicidal sensitive and resistant isolates of fungal pathogens (Gac *et al.*,1996; McKay, *et al*.,1998; McKay *et al*.,1999; Suwan *et al*., 2012). In this study, ITS sequences divided *A. porri* isolates into two groups, which is not correlated with fungicidal sensitivity, corresponded to pathological groups; moderately and highly aggressive isolates formed in one cluster and other cluster contained less aggressive and non- aggressive isolates. However, multilocus gene typing is required to map the aggressiveness and fungicidal sensitivity in addition to ITS sequences used in this study.

Thus, the diversity in *A. porri* isolates exhibiting different levels of aggressiveness identified in this study will be of great interest for the selection of onion genotypes showing different levels of resistance to PLB. A moderately aggressive isolate may be used to locate new sources of partial resistance, while highly aggressive isolate would be ideal for the final evaluation of hybrids carrying several resistance factors.

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Variation among guava (*Psidium guajava* L.) accessions in seed hardness and its association with fruit characteristics

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ABSTRACT

Guava is an important tropical fruit crop with considerable economic potential but to further increase popularity and demand, the soft seeded cultivars needs to be developed. Breeding for soft seeded cultivars is one of the major objectives of guava breeding programmes but limited research on seed hardness and its association with other fruit characters has been conducted. A study was carried out to determine the genotypic and phenotypic variation in and association among the fruit traits of weight, height, diameter, firmness and the seed characters of weight and number of seeds per fruit, fruit seed weight ratio, 100-seed weight and seed hardness. Trait values were estimated in 50 accessions grown under subtropical conditions. High genotypic (GCV) and phenotypic (PCV) coefficients of variation were found in all the traits studied. Estimates of heritability ranged from 0.499 (fruit firmness) to 0.988 (fruit seed weight ratio). All the traits except 100-seed weight, showed almost similar magnitude of genetic advance, and exceptionally high genetic advance for fruit weight, number of seeds per fruit and fruit: seed weight ratio. At genotypic and phenotypic levels, seed hardness was significantly and positively correlated with 100-seed weight of the fruit and inversely with number of seeds fruit^{-1}. In path analysis, residual values at genotypic (0.076) and phenotypic (0.268) levels were fairly low and indicate that variables included in this study had significant association with seed hardness**.** Correlation and path coefficient analysis revealed that 100-seed weight was the most important character for realizing improvement in seed hardness.

Key words: Guava, *Psidium guajava* L., seed hardness, fruit quality, fruit traits correlation

INTRODUCTION

Guava (*Psidium guajava* L.) of the Myrtle family (Myrtaceae), is one of the most important fruit tree crops in the tropics and subtropics and is commercially cultivated in more than 60 countries (Rajan *et al.*, 2007). In India, guava is particularly important because of its excellent nutritive value, wide adaptability and high productivity. Guava is an excellent source of Vitamin C and rich in nutraceuticals (Wilson III, 1980; Jimenez-Escrig *et al.*, 2001). Area under this crop has increased in India over time lot of variability is available due to propagation through seeds.

Fruits of the numerous guava varieties are extremely variable in shape, size and other characteristics (Yadava and Shanker, 2007). They may be globular, ovoid or pyriform and from 3 to 15 cm long. Skin may be greenish-white, yellowish, or pink and in taste, varieties range from sweet to tart, all with the characteristic musky flavour and odour of the guava more or less pronounced. Fruits also vary in the thickness of the fleshy mesocarp and in some varieties it is extremely thin. Most varieties bear numerous seeds embedded in the soft pulp at the center. Guava may be eaten fresh, made into a juice or nectar containing fruit pulp, or made into preserves, jam, jelly or paste. However, high content of hard and large sized seeds reduce the fruit quality in most of the guava varieties (Negi and Rajan, 2007).

Seed content and hardness in guava are determinants of fruit quality and vary with cultivar. There are some understandings that pink flesh colour may correlate with hardness of seeds but such correlations may not hold true for all the guava cultivars. Few studies have been conducted on the variability in seed characteristics (Rajan *et al.*, 2005). The high genetic coefficient of variation and heritability estimates associated with greater genetic advance as percent of mean were recorded for pulp: seed weight ratio, 100-seed weight and number of seeds per fruit (Rajan *et al.*, 2005). However, information on these estimates in relation to seed hardness and their association with fruit characters is not available. Accordingly, in this study heritability, genetic advance, correlation and path coefficient are evaluated with the objective of selecting the genotypes comprising few and soft seeds. Path coefficient analysis was done to ascertain the direct and indirect contribution of fruit and seed characters towards seed hardness in guava genotypes.

MATERIALS AND METHODS

Plant material

Fifty guava accessions available in the field gene bank (FGB) situated at the Central Institute for Subtropical Horticulture, Lucknow, India (26.85 °N latitude and 80.90 °E longitude and altitude of 128 m from mean sea level) were evaluated for seed characters during 2007 and 2008. The plants were maintained under uniform cultural conditions in the Indo-Gangatic plains. It has a subtropical climate with cool winter, dry and hot summer and intense rainy seasons. Three clonally multiplied trees of each accession replicated in randomized complete block design, were used for collection of fruit samples. In each replication, seeds were extracted from random fruits collected from four directions and outer part of the tree canopy. Thus 12 fruits of each accession and mean of four fruits per replication was used for data analysis. Fruits were harvested at colour break stage of the exocarp and weighed individually. Seeds were extracted from individual fruit, cleaned and air-dried for a week before their assessment.

Fruit and seed characters

Weight of fruit (FW) and seed weight per fruit (SWPF) were determined with a digital balance (Mettler AJ 100) and fruit: seed weight ratio (FSWR) was calculated. Diameter (FD) and height (FH) of fruit were measured at maximum diameter with a standard calipers. 100-seed weight (100SW) was measured by weighing 100-seeds on electronic balance (Mettler AJ 100) with 0.0001g least count. Image analysis was performed for counting the seeds. For each fruit sample, extracted seeds were spreaded on black paper without touching each other. Seeds were digitally photographed and saved in TIFF format for image analysis. Number of seeds per fruit (NSPF) were counted with the help of JavaScript image analysis software, ImageJ (http://rsb.info.nih.gov/ij/index.html), using digital image. Seed hardness (SH) and fruit firmness (FF) were measured by Mecmesin Basic Force Gauge (BFG 1000N, England) using an 8 mm probe and expressed as kg cm^{-2}. Maximum fracture force required to crack a seed was recorded on 20 seeds per fruit. A thin slice of skin (about 2-mm thick and slightly larger diameter than the probe) was removed from the position with largest fruit diameter before the firmness measurement.

Biometrical analysis

The experimental data was analysed in Standard Randomized Complete Block with three replications. Mean sum of squares were calculated using ANOVA over three replications for genotypic and phenotypic coefficient of variation. Genotypic and phenotypic coefficient of variation for various characters were computed as per Burton and De Vane (1953), and estimates of heritability (broad-sense) and genetic advance (Johnson *et al.,* 1955; Allard, 1960; Hansen, 1963; Falconer, 1989) were calculated as follows:

$$\sigma_g^2 = \frac{(Mg - Me)}{r}$$

$$\sigma_p^2 = \sigma_g^2 + \sigma_e^2$$

$ó^2_e = Me$

$$\text{GCV} = \frac{\sqrt{\sigma_g^2}}{\bar{X}} \times 100$$

$$\text{PCV} = \frac{\sqrt{\sigma_p^2}}{\bar{X}} \times 100$$

$$h_b^2 = \frac{\sigma_g^2}{\sigma_p^2}$$

$GA = ih^2 \sigma_p$

Where,

GCV = genotypic coefficient of variation

PCV = phenotypic coefficient of variation

Mg = mean sum of square due to genotypes, *Me* = mean sum of square due to error, r = number of replications, GA = Genetic advance and $\bar{X}$ is the population mean.

h^2_b is heritability in broad sense, $ó^2_g$, $ó^2_p$ and $ó^2_e$ are the genotypic, phenotypic and environmental variance, respectively, and *i* is the standardized selection differential at selection intensity of 5%.

Genetic (r_g) and phenotypic (r_F) correlations between two x and y traits were analyzed from the mean value of various accessions using the procedure outlined by Kempthorne (1973) and Miller *et al.* (1958) as follows:

$$\hat{r}_g(x,y) = \frac{\hat{COV}_g(x,y)}{\sqrt{\hat{\sigma}_g^2(x)}\sqrt{\hat{\sigma}_g^2(y)}}$$

$$\hat{r}_p(x,y) = \frac{\hat{COV}_p(x,y)}{\sqrt{\hat{\sigma}_F^2(x)}\sqrt{\hat{\sigma}_F^2(y)}}$$

Path coefficients between seed hardness and all possible

combinations of variables were calculated as per method of Dewey and Lu (1959). Path analysis is a special type of multivariate analysis, which enables us to provide so-called correlation analysis according to the following equation:

$$r_{yi} = \hat{p}_{yi} + \sum_{i'=1, i' \neq i}^{k} r_{ii'} \hat{p}_{yi} \quad \text{for } (i \neq i')$$

Where: r_{yi} is correlation coefficient between the *i*-th causal variable (X_i) and effect variable (y), $r_{ii'}$ is simple correlation coefficient between the *i*-th and the *i'*-th causal variable, $\hat{p}_{yi}$ is path coefficient (direct effect) of the *i*-th causal variable (X_i), $r_{ii} \hat{p}_{yi}$ is called indirect effect of the *i*-th causal variable via the *i'*-th causal variable.

We made path coefficient analysis between seed hardness (effect variable) and eight causal variables: fruit weight (FW), fruit height (FH), fruit diameter (FD), fruit firmness (FF), seed weight per fruit (SWPF), number of seed per fruit (NSPF), fruit seed weight ratio (FSWR), 100-seed weight (100SW) using data from both study years. They were based on genotypic and phenotypic correlation coefficients for means of the full-sib population.

RESULTS AND DISCUSSION

Fruit and seed characters *viz.*, fruit weight, height, diameter, firmness and seed weight and number of seeds per fruit, fruit seed weight ratio, 100-seed weight and seed hardness differed significantly among the accessions (Table I).

High genotypic (GCV) and phenotypic (PCV) coefficient of variation estimates were recorded for all the traits. However, the genotypic coefficient of variation (GCV) was maximum for fruit: seed weight ratio (230.79%) followed by fruit weight (53.78%) number of seeds per fruit (49.37%), seed weight per fruit (46.27%), 100-seed weight (30.00%) seed hardness (25.04%), fruit firmness (19.87%), fruit height (19.21%) and diameter (18.68%). The characters showing high GCV indicates that they were highly influenced by the genotypic components. The estimates of phenotypic coefficient of variation (PCV) ranged from 20.01 (average fruit diameter) to 232.14% (fruit: seed weight ratio). Nevertheless, the difference between GCV and PCV was small for all the traits except fruit firmness, indicating that genetic factors rather than the environment exerted the major influence on the variation and these traits are valuable for crop improvement. Environmental features such as storage temperature as well as stage of maturity are other important factors influencing fruit firmness (Thaipong and Boonprakob, 2005). Such influences indicated the wide gap between GCV and PCV for fruit firmness. Textural attributes of fruits are related to the structural, physiological, biochemical characteristics of the living cells and change with the ripening of the fruit (Harker *et al.*, 1997).

Most of the traits had high heritability estimates, ranging from 0.499 to 0.988 (Table 1). High broad sense heritability for all the traits is another significant indicator that may be used as selection criteria even in variable environmental conditions (Mahon, 1983 and Messina, 1993). A wide range of values from 0.61 to 365.89 was observed for genetic advance. All the traits, except 100-seed weight showed almost similar magnitude of genetic advance, ignoring the exceptionally high for fruit weight, number of seeds per fruit and fruit seed weight ratio. Thus high genetic coefficient of variation (GCV) and heritability estimates indicated that these three characters had additive gene effect and therefore effective selection can be made for these characters.

As far as interrelationship is concerned, seed hardness showed positive correlations with 100-seed weight but was negatively associated with fruit firmness, number of seeds per fruit at both genotypic and phenotypic levels (Table 2). Fruit weight showed positive genetic and phenotypic correlations with fruit height and diameter and with weight and number of seeds per fruit. Height and diameter of the fruit exhibited significant positive correlation with weight and number of seeds per fruit. Fruit firmness had positive correlation with number of seeds per fruit and negative association with seed hardness. Seed weight per fruit showed significant positive relationship with number of seeds per fruit and negative association with fruit: seed weight ratio and 100-seed weight. Number of seeds per fruit also showed negative association with 100-seed weight. Correlations among different traits can be exploited to select a desired plant type.

However, these correlation coefficient values sometimes do not serve as a reliable tool thus selections based on these estimates give meager or no response to selection. Because a trait showing positive association may not have direct effect on seed hardness but may contribute to seed hardness indirectly via other traits. The genotypic (0.076) residual value was fairly low, which apparently showed that variables included in this study had significant contribution. At phenotypic level, high residual value (0.268) indicated the limitation of traits included in the study, which need to be supplemented with more morphological characters to illustrate the whole range of variation. Considering such opinion, direct positive effects of fruit height, number of seeds per fruit, seed weight per fruit and 100-seed weight and negative direct effects of weight, diameter and firmness of fruit and fruit seed weight ratio were observed on seed hardness (Table 3). The study revealed that the positive

Table 1: *Estimates of genotypic (GCV) and phenotypic (PCV) coefficients of variation, heritability (h^2_b) and genetic advance as percent of mean*

Characters	GCV(%)	PCV(%)	(h^2_b)	Genetic advance as percent of mean	F$
Fruit weight	53.78	57.23	0.883	162.57	23.63**
Fruit height	19.21	20.78	0.854	2.33	18.54**
Fruit diameter	18.68	20.01	0.871	2.26	21.31**
Fruit firmness	19.87	28.11	0.499	1.05	03.99**
Seed weight per fruit	46.27	50.77	0.831	2.79	15.72**
Number of seed per fruit	49.37	53.67	0.846	306.05	255.98**
Fruit seed weight ratio	230.79	232.14	0.988	365.89	17.52**
100-seed weight	30.00	33.65	0.795	0.61	12.62**
Seed hardness	25.04	27.55	0.826	5.53	15.20**

**= Significant at p≤0.01.
$ Calculated "F" value from analysis of variance

Table 2: *Genotypic (G) and Phenotypic (P) correlation coefficient among 8 characters in 50 guava genotypes*

Characters	FW	FH	FD	FF	SWPF	FSWR	NSPF	100SW	SH
FW		0.719**	0.893**	-0.019	0.727**	-0.027	0.482**	-0.049	-0.103
FH	0.722**		0.642**	0.016	0.434**	-0.026	0.233	-0.034	0.018
F D	0.890**	0.641**		0.198	0.773**	0.004	0.613**	-0.232	-0.301**
FF	0.015	0.052	0.137		0.204	0.172	0.334*	-0.426**	-0.546**
SWPF	0.678**	0.401**	0.712**	0.183		-0.344*	0.892**	-0.378**	-0.270*
FSWR	-0.017	-0.013	0.015	0.108	-0.322*		-0.344*	0.422**	-0.005
NSPF	0.440**	0.218	0.556**	0.248	0.859**	-0.324*		-0.667**	-0.551**
100SW	-0.004	0.004	-0.166	-0.263*	-0.280*	0.395**	-0.617**		0.856**
SH	-0.032	0.049	-0.206*	-0.338**	-0.176	-0.003	-0.464**	0.775**	

*= Significant at p≤0.05, **= Significant at p≤0.01
Above diagonal values represent genotypic correlation coefficient whereas below diagonal show phenotypic correlation coefficients
FW= fruit weight, FH= fruit height, FD= fruit diameter, FF= fruit firmness, SWPF= seed weight per fruit, FSWR= fruit: seed weight ratio, NSPF= number of seed per fruit, 100SW= 100-seed weight, SH= seed hardness, G= genotypic correlation, P= phenotypic correlation

Table 3: *Path coefficient values estimated for seed hardness and other characters*

Pathways of associations	Genotypic	Phenotypic
Seed hardness vs. fruit weight		
Direct effect	-0.283	-0.073
Indirect effect via fruit height	0.159	0.114
Indirect effect via fruit diameter	-0.032	-0.083
Indirect effect via fruit firmness	0.001	-0.001
Indirect effect via seed weight per fruit	0.041	-0.051
Indirect effect via fruit seed weight ratio	0.011	0.006
Indirect effect via number of seeds per fruit	0.052	0.069
Indirect effect via 100-seed weight	-0.052	-0.004
Correlation, r_{19}	-0.103	-0.032
Seed hardness vs. fruit height		
Direct effect	0.221	0.158
Indirect effect via fruit weight	-0.203	-0.053
Indirect effect via fruit diameter	-0.023	-0.060
Indirect effect via fruit firmness	-0.001	-0.004
Indirect effect via seed weight per fruit	0.025	-0.030
Indirect effect via fruit seed weight ratio	0.010	0.004
Indirect effect via number of seeds per fruit	0.025	0.029
Indirect effect via 100-seed weight	-0.036	0.003
Correlation, r_{29}	0.018	0.049
Seed hardness vs. fruit diameter		

Contd.

Pathways of associations	Genotypic	Phenotypic
Direct effect	-0.036	-0.093
Indirect effect via fruit weight	-0.253	-0.065
Indirect effect via fruit height	0.142	0.102
Indirect effect via fruit firmness	-0.015	-0.009
Indirect effect via seed weight per fruit	0.044	-0.054
Indirect effect via fruit seed weight ratio	-0.001	-0.005
Indirect effect via number of seeds per fruit	0.066	0.075
Indirect effect via 100-seed weight	-0.247	-0.156
Correlation, r_{39}	-0.301**	-0.206*
Seed hardness vs. fruit firmness		
Direct effect	-0.078	-0.068
Indirect effect via fruit weight	0.005	-0.001
Indirect effect via fruit height	0.003	0.008
Indirect effect via fruit diameter	-0.007	-0.013
Indirect effect via seed weight per fruit	0.012	-0.014
Indirect effect via fruit seed weight ratio	-0.066	-0.037
Indirect effect via number of seed per fruit	0.036	0.034
Indirect effect via 100-seed weight	-0.452	-0.247
Correlation, r_{49}	-0.546**	-0.338**
Seed hardness vs. seed weight per fruit		
Direct effect	0.057	-0.075
Indirect effect via fruit weight	-0.206	-0.049
Indirect effect via fruit height	0.096	0.064
Indirect effect via fruit diameter	-0.028	-0.066
Indirect effect via fruit firmness	-0.016	-0.012
Indirect effect via fruit seed weight ratio	0.133	0.111
Indirect effect via number of seed per fruit	0.096	0.116
Indirect effect via 100-seed weight	-0.402	-0.263
Correlation, r_{59}	-0.270*	-0.176
Seed hardness vs. fruit seed weight ratio		
Direct effect	-0.386	-0.344
Indirect effect via fruit weight	0.008	0.001
Indirect effect via fruit height	-0.006	-0.002
Indirect effect via fruit diameter	0.000	-0.001
Indirect effect via fruit firmness	-0.013	-0.007
Indirect effect via seed weight per fruit	-0.020	0.024
Indirect effect via number of seed per fruit	-0.037	-0.044
Indirect effect via 100-seed weight	0.447	0.371
Correlation, r_{69}	-0.005	-0.003
Seed hardness vs. number of seed per fruit		
Direct effect	0.107	0.135
Indirect effect via fruit weight	-0.136	-0.032
Indirect effect via fruit height	0.052	0.034
Indirect effect via fruit diameter	-0.022	-0.052
Indirect effect via fruit firmness	-0.026	-0.017
Indirect effect via seed weight per fruit	0.051	-0.065
Indirect effect via fruit seed weight ratio	0.133	0.112
Indirect effect via 100-seed weight	-0.709	-0.579
Correlation, r_{79}	-0.551**	-0.464**
Seed hardness vs. 100-seed weight		
Direct effect	1.063	0.939
Indirect effect via fruit weight	0.014	0.000
Indirect effect via fruit height	-0.007	0.001
Indirect effect via fruit diameter	0.008	0.015
Indirect effect via fruit firmness	0.033	0.018
Indirect effect via seed weight per fruit	-0.022	0.021
Indirect effect via fruit seed weight ratio	-0.163	-0.136
Indirect effect via number of seed per fruit	-0.071	-0.083
Correlation, r_{89}	0.856**	0.775**

*= Significant at $p \leq 0.05$, **= Significant at $p \leq 0.01$

direct effects reflect the impact of number of seeds per fruit on seed hardness as showed significantly negative association with seed hardness. The 100-seed weight exhibited maximum positive direct effect on seed hardness.

Such positive direct effect established the fact that 100-seed weight exhibit large seed in the fruit. Thus it reveals that increase in 100-seed weight directly related to seed hardness. Despite that boldness attributed to size and/or specific gravity of the seed which reflects the hardness. These findings confirmed the reports of Bisla and Daulta (1987), Prajapati *et al.* (1996) and Pareek and Dhaka (2003), regarding the direct and indirect effects of characters on fruit yield through path coefficient analysis in ber (*Zizyphus* spp.). However, 100-seed weight will be better parameters for correlating seed hardness. Contrary to the findings of Gohil *et al.* (2006), our results indicates that pulp weight has weak relationship with seed hardness. Gohil *et al.* (2006) reported that pulp weight and seed weight had positive direct effects on seed hardness. Fruit size exhibited positive direct effects on pulp weight and seed weight in guava.

Thus it may be concluded that wide variation exist in seed hardness associated with fruit and seed characteristics of guava. Heritability and genetic advance as percent of mean for fruit: seed weight ratio, fruit weight, number of seeds fruit^{-1}, seed hardness and 100-seed weight indicated scope of effective selections. Correlation and path coefficient analysis revealed that 100-seed weight was the most important character for realizing improvement for seed hardness in guava genotypes.

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Evaluation of critical limits and development of leaf nutrient standards in litchi (*Litchi chinensis* Sonn.)

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ABSTRACT

An experiment was conducted to evaluate existing critical limits and develop leaf nutrient standards in litchi. Thirty orchards with 20 ±2 years old trees planted with the cultivar Shahi (15 orchards) and China (15 orchards) in different districts of Bihar and Jharkhand, India were selected for the study. In order to develop soil and leaf nutrient standard, relations were exhibited between leaf nutrients content and yield. There was a little difference in values of leaf nutrient among cultivars Shahi and China, but the differences were not enough to suggest that different standards are required for different cultivars as the differences were due to nutrient uptake and not due to requirements. Thus, standard ranges have to allow tolerance to varietal variability. Leaf nutrient standards in litchi have been developed for the first time in India and would be used as a guide for litchi orchards. Among the nutrients N, P, Ca, Mg, Zn, Fe, B appeared to be more essential, which could be monitored, based on the leaf nutrient content. However, the standard would need refinement based on response to applied fertilizer. The critical value for the nutrients for leaf nutrient standard were N-1.75%, P-0.17%, K-0.80%, S-0.25%, Ca-0.90%, Mg-0.60%, Fe-100 ppm, Mn-120 ppm, Zn-28 ppm, Cu-30 ppm, B-29 ppm.

Key words: Litchi, leaf nutrient standard, soil nutrient standard, yield, macronutrient, micronutrient, critical limits.

INTRODUCTION

Litchi (*Litchi chinensis* Sonn.), is an important fruit crop of India, has gained its importance globally due to its appearance, taste and quality. Commercial industries have developed in several countries like Taiwan, Thailand, India, South Africa and Australia (Menzel and Simpson, 1987). In India, its commercial cultivation is done in Bihar, Jharkhand, West Bengal, Uttaranchal and some part of north India out of which more than 56 percent production of litchi comes from Bihar and Jharkhand states of the total production of litchi in India. Therefore, the study was conducted in these two states to evaluate existing critical limits of leaf nutrient standards in Litchi. Balanced and adequate nutrition is essential for the plant growth and yield to their maximum potential. No universal nutrition program seems to be available for litchi, and poor nutrition is likely to be one of the major factors contributing to fluctuating yields (Menzel and Simpson, 1987). Therefore, orchards were surveyed and thirty orchards were selected in these two states which had 20 ±2 years old trees of cv. Shahi and China.

MATERIALS AND METHODS

The study was conducted on 20 ±2 years old trees of cv. Shahi and China grown in different districts of Bihar and Jharkhand. A total of 30 orchards planted with the cultivar Shahi (15 orchards) and China (15 orchards) were selected in different districts of Bihar and Jharkhand, for the study. All these orchards were planted in square system of planting at the spacing of 8-8.5 m. Package of practices, with respect to nutrition varied from orchard to orchard. To assess the leaf nutrient status the autumn (September) flush was selected as it was the most fruitful of the flushes (Kotur and Singh, 1993; Singh *et al*., 2010b). In order to asses the variability in litchi orchards with respect to soil characteristics and also quality of fruits produced, soil samples collected from different litchi orchards at the depth of 15-30 cms were analysed for pH, Electrical Conductivity (EC), organic carbon (OC), P, K, Ca, Mg, Fe, Mn, Zn and Cu content. While for the leaf samples, second pair of leaflets from tip of the terminal, 6-7 months old, from all directions and at mid height (6-7ft.) of the tree (Singh and Chadha, 2009) which were 40 in number were collected

from ten healthy trees of each orchard and were marked. The leaves collected from all the ten trees were pooled to get representative sample of an orchard. These samples were decontaminated and then dried at 60 ± 1°C in hot air oven till constant weight. Total nitrogen was analysed by Kjeltech-2300 auto analyzer using 40% NaOH, 1% boric acid and 0.1 N HCL. Samples were wet digested with diacid mixture of nitric acid and perchloric acid in the ratio of 9:4. Vanado-molybdate colour reaction method was used for estimation of phosphorus using spectrophotometer. Potassium content was estimated using flame photometer. To determine sulphur content Turbid biometric method was used using Spectrophotometer, while Ca, Mg, Fe, Mn, Zn and Cu was determined using Atomic Absorption Spectrophotometer (AAS).

RESULTS AND DISCUSSION

The soil and leaf samples of the different orchard selected for the study from both the states were analysed. The soil samples at different depths were examined for pH, EC, OC, P, K, S, Ca, Mg, Fe, Mn, Zn and Cu content for all the orchards of cultivars Shahi and China (Table 1). The yield and quality of fruits and physical qualities of different orchards for cultivar Shahi and China were also assessed (Table 2). Similarly, even the leaf samples were analysed for N, P, K, S, Ca, Mg, Fe, Mn, Zn and Cu content for cultivars Shahi (Table 3) and China (Table 4).

Table 1: Characteristics of soils of Litchi orchards for cultivars Shahi and China

	Shahi	China
pH	5.06 – 8.94	4.82 – 8.63
EC (dsm^{-1})	0.04 – 0.47	0.05 – 0.17
OC (%)	0.18 – 0.41	0.18 – 0.50
P (ppm)	0.51 – 3.25	0.60 – 2.00
K (ppm)	41.00 – 99.51	34.00 – 85.5
S (ppm)	32.10 – 51.65	19.99 – 53.31
Ca (ppm)	271.61 – 2040.11	488.20 – 2137.33
Mg (ppm)	10.81 – 34.71	12.20 – 32.78
Fe (ppm)	4.39 – 15.43	6.84 – 11.40
Mn (ppm)	21.83 – 38.20	22.54 – 35.48
Zn (ppm)	0.32 – 0.70	0.31 – 0.66
Cu (ppm)	0.85 – 2.94	1.13 – 3.19

Soil nutrient content

The soil status, in orchards of the cultivars varied depending on the type of soil and geographical location. The pH value ranged between 5.06 to 8.9 which confirms the perception that litchi tree has wide adaptability of soil pH (Arnon and Johnson, 1942). Litchi is grown successfully in the pH range of 7.5-8.5 in calcareous soils of Bihar, while in Jharkhand state it is grown in the laterite soil having pH of 5.0 to 5.5 is in confirmation with the study of Rai *et al.*, 2001. pH of soil directly or indirectly influence availability of various nutrients which ultimately influence yield and quality. OC content in the soil was related with the age of orchards which indicated that leaf residues which are more in older orchards had influence on soil OC content. Nutrient K content of the soil was higher in soils of Jharkhand when compared to Bihar, it increased as the pH decrease upto the pH value 5.0 and continued to decrease above 7.0 pH, this confirms the findings of Menzel and Kirkby, 1979. Soil nutrient P and S content was not dependent on the geographic location of the soil type, and varied from orchard to orchard. Soil Ca content was highly variable among the orchards but higher Ca content was observed in litchi orchards of Bihar which has been considered to be conducive for better growth and yield and lime application is essential in soils with low Ca content (Singh and Babita, 2002). Soil Mg, Fe, Mn, Zn content could not establish any definite trend due to the geographical location of the soil.

Variation in yield and quality

Fruit yield, in terms of weight per plant varied from orchards to orchards for both the cultivars. Variation could be attributed to the difference in nutritional case of litchi plants (Rao *et al.*, 1985). Since soils of orchards varied significantly for soil pH, EC and nutrient content, yield variation was noticed which was reflected in leaf nutrient content also. Orchards in alluvial soil of Bihar had higher yield, and soil pH was found to influence the production (Kanwar, 2002). Therefore, variability in yield, among the orchards for both the cultivars could be attributed to soil condition and management practices. Fruit weight varied significantly among the orchards, mean value of average fruit weight varied from 18.19 to 23.10 g and 17.35-19.85 g for cultivar Shahi and China respectively. There was a wide variation for TSS content which ranged between 19.92-23.65 °Brix and 19.08-20.63 °Brix for cv. Shahi and China respectively (Table 2).

Table 2: Yield and properties of fruits of cultivars Shahi and China

	Shahi	China
Yield/ Tree (kg)	41.32 – 132.65	42.85 – 133.72
Average Fruit weight (g)	18.19 – 23.10	17.34 – 19.74
TSS (°Brix)	19.92 – 23.65	19.08 – 20.65

Studies on leaf nutrient content

The leaf N and P content was significantly influenced with the physiography and management practices adopted but there was significant variation among the orchards indicating that leaf nutrient content is influenced significantly due to management practices adopted by the

farmers. Leaf K content was significantly influenced by physico-chemical properties of the soil apart from the management practices. Although there appears to be a trend in leaf K content being influenced by the soil but there were significant variations among the orchards for leaf K content even in the nearby orchards. Leaf K content was lower in the orchards with high Ca content and higher with low Ca content in the soil. Interestingly, leaf K content appears to have relation with soil pH and EC besides availability of K in the soil. Leaf S content was influenced due to the soil conditions and management practices and leaf S content increased with increase in soil pH. Leaf Ca content in litchi orchards for cultivar Shahi was significantly influenced due to the location and the type of soil of the orchards and was very low in the orchards located in acidic soils. The orchards located in acidic soil had lower leaf Mg content compared to orchards having alluvial soil with pH above neutral. Leaf Fe, Mn and Zn content was invariably low in the orchards located in acidic soils and comparatively higher in alluvial soils and was also significantly influenced due to the location of the orchards and varied significantly from orchard to orchard. Leaf Cu content was not influenced

Table 3: Leaf nutrient content in different litchi orchards of cultivar Shahi

Orchard no.	N (%)	P (%)	K (%)	S (%)	Ca (%)	Mg (%)	Fe (ppm)	Mn (ppm)	Zn (ppm)	Cu (ppm)	B (ppm)
14M	1.67	0.17	0.66	0.27	1.01	0.67	110.74	144.83	34.78	23.08	25.85
16M	1.74	0.15	0.61	0.29	1.00	0.70	106.20	133.27	30.61	26.41	24.01
18M	1.67	0.17	0.69	0.30	0.99	0.64	119.13	128.61	33.56	36.44	29.83
22M	1.87	0.13	0.56	0.22	0.85	0.63	108.58	110.95	32.00	21.27	22.85
25S	1.83	0.15	0.59	0.23	0.81	0.58	117.31	111.90	29.00	23.78	28.34
1R	1.55	0.10	1.08	0.35	0.41	0.37	99.10	97.14	23.36	25.32	15.59
4R	1.53	0.10	1.13	0.33	0.42	0.36	69.09	97.47	22.68	23.27	17.01
11M	1.67	0.15	0.63	0.25	0.78	0.64	118.93	118.61	25.93	33.50	23.28
21M	1.79	0.11	0.58	0.28	0.85	0.64	108.55	113.60	24.81	24.42	19.35
30S	1.52	0.18	0.78	0.22	0.79	0.62	110.42	112.07	23.78	22.00	28.20
5R	1.35	0.10	1.20	0.35	0.47	0.39	68.53	99.04	18.72	35.88	17.74
7V	1.48	0.13	0.79	0.26	0.72	0.57	104.52	104.94	20.43	24.86	24.43
8V	1.38	0.12	0.85	0.27	0.71	0.54	108.78	115.02	19.60	26.26	20.94
28S	1.50	0.13	0.78	0.28	0.70	0.61	113.79	112.23	20.64	26.97	24.90
29S	1.44	0.11	0.81	0.24	0.79	0.62	105.89	112.61	19.93	25.21	19.83
F test	**	**	**	**	**	**	**	**	**	**	**
SE m ±	0.03	0.01	0.02	0.03	0.01	0.02	2.10	2.03	0.32	1.28	0.70
LSD (5%)	0.05	0.02	0.03	0.05	0.02	0.03	4.12	3.98	0.63	2.50	1.37
LSD (1%)	0.07	0.02	0.04	0.07	0.02	0.04	5.41	5.24	0.83	3.29	1.79

Table 4: Leaf nutrient content in different litchi orchards cultivar China

Orchard no.	N (%)	P (%)	K (%)	S (%)	Ca (%)	Mg (%)	Fe (ppm)	Mn (ppm)	Zn (ppm)	Cu (ppm)	B (ppm)
12M	1.76	0.13	0.66	0.29	0.77	0.62	104.30	118.10	26.58	26.07	27.74
13M	1.79	0.13	0.62	0.30	0.76	0.61	105.94	113.98	31.64	27.41	26.25
15M	1.78	0.16	0.63	0.28	0.98	0.63	129.51	144.48	24.54	38.68	31.26
17M	1.89	0.20	0.55	0.27	0.85	0.61	123.88	134.60	29.30	25.90	35.26
23M	1.98	0.17	0.53	0.27	0.82	0.60	107.50	113.69	21.55	26.43	30.61
2R	1.61	0.10	1.10	0.42	0.41	0.30	72.57	83.56	21.53	27.56	23.50
19M	1.60	0.12	1.17	0.28	0.98	0.62	116.94	131.79	20.57	27.64	26.26
20M	1.82	0.16	0.62	0.27	0.97	0.63	120.76	126.74	22.65	34.33	31.76
24M	1.84	0.17	0.59	0.30	0.85	0.59	108.06	118.14	24.15	27.39	30.93
26S	1.56	0.18	0.80	0.23	0.80	0.61	105.65	116.92	19.46	26.72	31.27
3R	1.48	0.10	1.15	0.41	0.40	0.29	69.43	85.03	16.57	38.48	22.73
6V	1.61	0.14	0.72	0.35	0.72	0.41	102.91	111.15	21.13	27.93	25.72
9V	1.51	0.13	0.80	0.31	0.72	0.43	92.93	109.92	20.56	28.84	28.37
10V	1.58	0.14	0.74	0.26	0.68	0.49	97.63	108.69	19.48	28.60	31.59
27S	1.46	0.12	0.83	0.22	0.77	0.57	104.95	115.39	18.02	28.36	26.39
F test	**	**	**	**	**	**	**	**	**	**	**
SE m ±	0.02	0.01	0.01	0.01	0.02	0.02	2.81	1.62	0.38	0.85	0.95
LSD (5%)	0.04	0.01	0.03	0.02	0.04	0.03	5.51	3.17	0.74	1.66	1.86
LSD (1%)	0.05	0.02	0.03	0.03	0.05	0.04	7.25	4.16	0.97	2.19	2.44

Table 5: Leaf nutrient standard values for litchi

Nutrients	Africa	Australia	Australia	India (Present Investigation)		
	(Cull, 1977)	*(Anon, 1983)*	*(Menzel et al., 1992)*	Range (max & min)	Critical range	Critical value
N %	1.3 – 1.4	1.3 – 1.4	1.6 – 1.7	1.33 – 2.00	1.7 – 1.90	1.75
P %	0.08 – 0.1	0.17 – 0.2	0.15 – 0.25	0.09 – 0.20	0.16 – 0.20	0.17
K %	1.0 -	0.8 – 1.2	0.85 – 0.95	0.50 – 1.21	0.70 – 0.90	0.80
S %	-	-	-	0.21 – 0.43	0.25 – 0.30	0.25
Ca %	1.5 – 2.5	0.56	0.5 – 0.55	0.38 – 1.02	0.80 – 1.0	0.90
Mg %	0.4 – 0.7	0.21	0.35 – 0.45	0.28 – 0.65	0.50 – 0.68	0.60
Fe ppm	50 – 200	50 – 200	50 – 100	67 – 120	95 – 120	100
Mn ppm	50 – 200	30 – 500	180 – 210	90 – 150	105 – 130	120
Zn ppm	15	30 – 150	15 – 20	16 – 35	25 – 30	28
Cu ppm	10	5 – 15	5 – 10	20 – 40	26 – 35	30
B ppm	27 – 75	50 – 100	20 – 25	15 – 35	22 – 30	29

by the geographic regions as seen for many of the mineral elements, of the orchards but was influenced by the management practices followed for the orchardist. Orchards located in red laterite soils having lower pH had invariably very low B content compared to the orchards located in alluvial soils. It is also evident from the investigation that leaf B content was influenced both by locations of the orchards and also by management practices adopted within the district. The leaf nutrient content for both the cultivars are presented in Table 3 and 4. No definite relationship could be established for leaf nutrient content to orchards. This is because of variation among agro-climatic conditions, soil series, management practices as well as meteorological parameter, which differ from orchard to orchard.

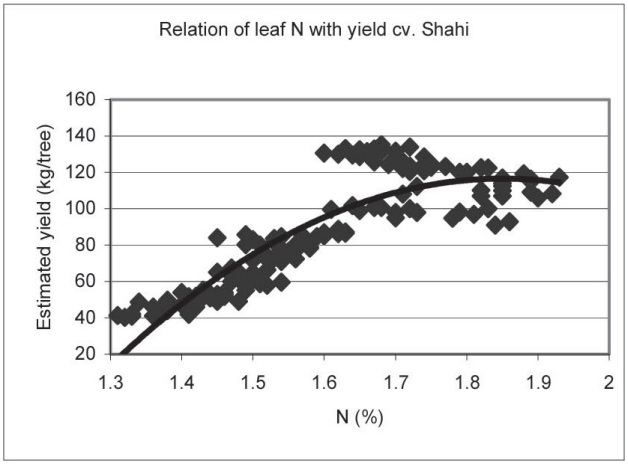
Interrelations of the leaf nutrient status with the yield

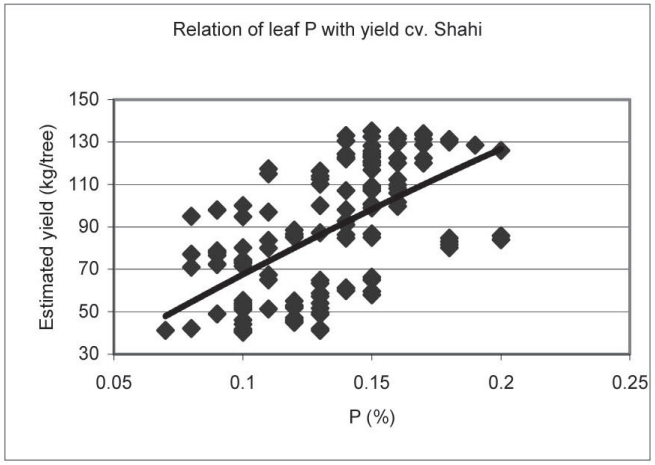
The interrelations of the leaf nutrient content with yield for cvs. Shahi and China are depicted in Fig. 1a, 1b and 2a, 2b respectively. It is concluded from the study that the yield increases with the increase in leaf N and Zn, and declines after reaching optimum level, while for leaf P, Ca, Mg, Fe, Mn and Cu content the trend was not clear. The yield declined with the increase in leaf K and leaf S. This trend was observed for both the cultivars cultivar Shahi and China for both the years of observations.

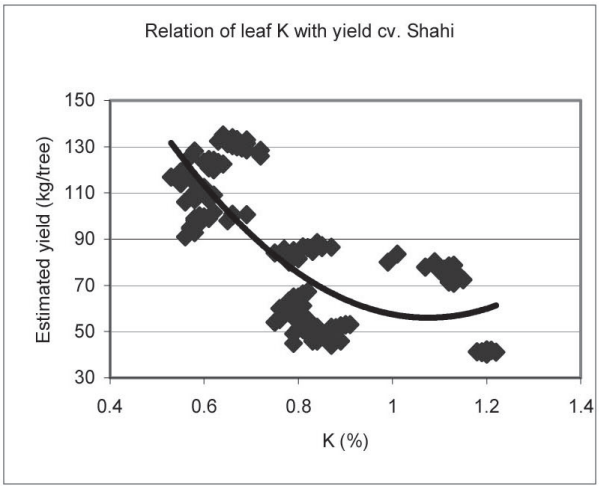
Development of leaf nutrient standards in litchi

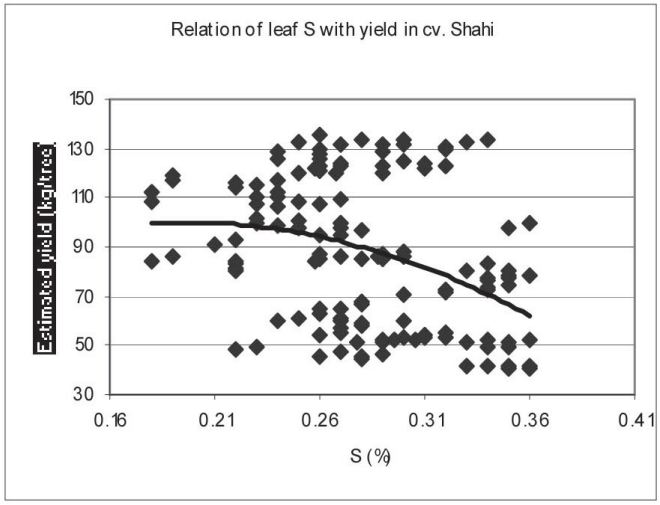
While interpreting the result of leaf analysis consideration of single element will have limited values. Understanding of the effect of the elements on one another is also desirable due to the synergistic and antagonistic influence on mineral content. Therefore it was observed that higher N level had suppressive effect on K, S, Fe and Mn while increased K had suppressive effect on Ca, Mg, N and Zn. Antagonistic and synergistic effect of element in the present investigation is in conformity with the result of Smith (1966). The leaf nutrient standard values derived therefore can be used for nutritional management in litchi, which would require refinement based on field trials to achieve targeted yield and quality of fruits, using appropriate sampling technique and interpretation of derived nutrient values. Variation in soil and leaf nutrient content is attributed to soil condition, management practices adopted and varietal differences (Rao *et. al.,* 1985). The observations that were recorded from the survey of orchards, are used to work out the leaf nutrient standards based on the relations established with soil nutrient content, leaf nutrient content with yield and quality of fruits. In order to develop leaf nutrient standard relations were exhibited between leaf nutrients content and yield. Through scatter diagram and regression studies. Leaf nutrients, which had maximum yield values, were taken as values of leaf to obtain optimum yield. The maximum and minimum range, critical range and critical values are depicted in Table 5.

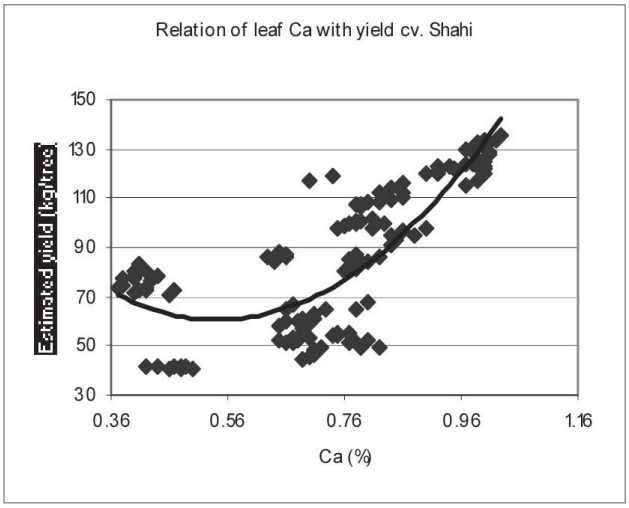
Leaf nutrient standards of litchi developed in the present investigation, derived from all feasible angles are tentative values. (Cull, 1977; Anon, 1983 and Menzel *et al*., 1972). The tentative values derived in present investigation are closer to values reported by Menzel *et. al*. (1992). Leece *et. al.,* (1971) observed that standards developed in different countries could be similar if standard samples are taken. Thus, standard developed will have larger application. There was a little difference in values of leaf nutrient among cultivar Shahi and China, but the differences were not enough to suggest that different standards are required for different cultivars, as the differences were due to nutrient uptake not due to requirements. Thus, standard ranges have to allow tolerance to varietal variability. It is further concluded that leaf nutrient standard developed in this study could be adopted for nutrient management in litchi. For appropriate diagnostic sampling 4-7 month old, 40 numbers of leaflets from the middle of the canopy from non-fruiting











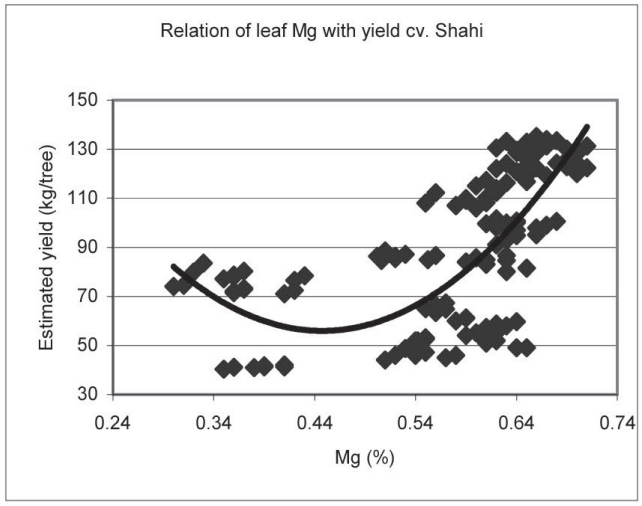
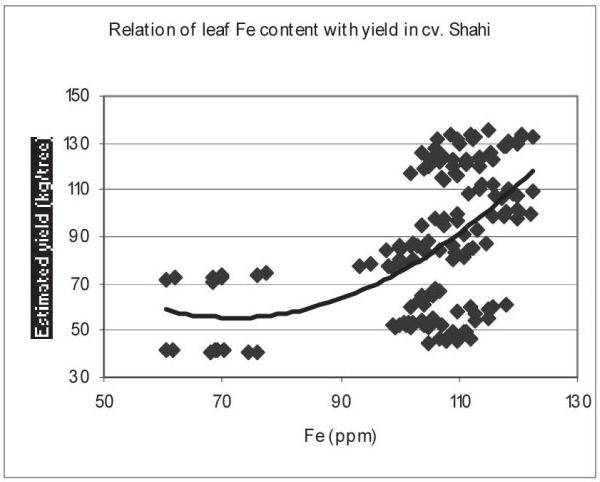
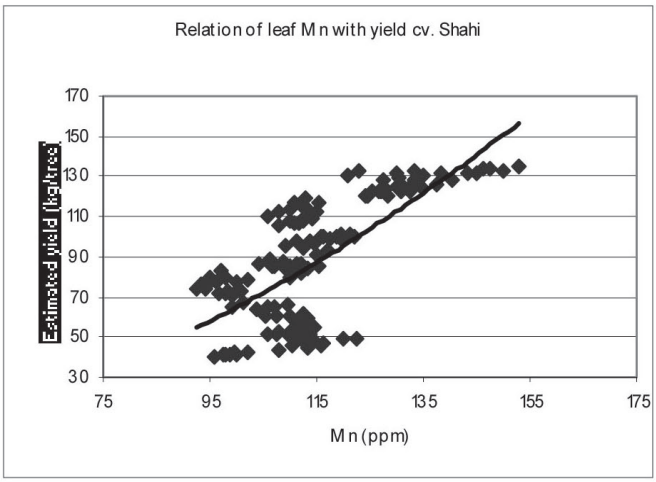
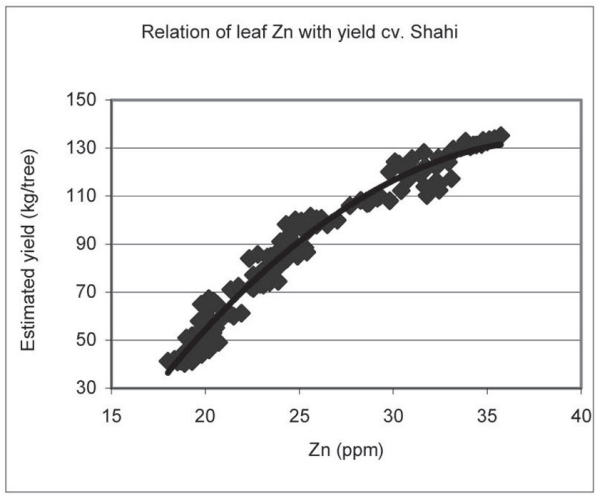
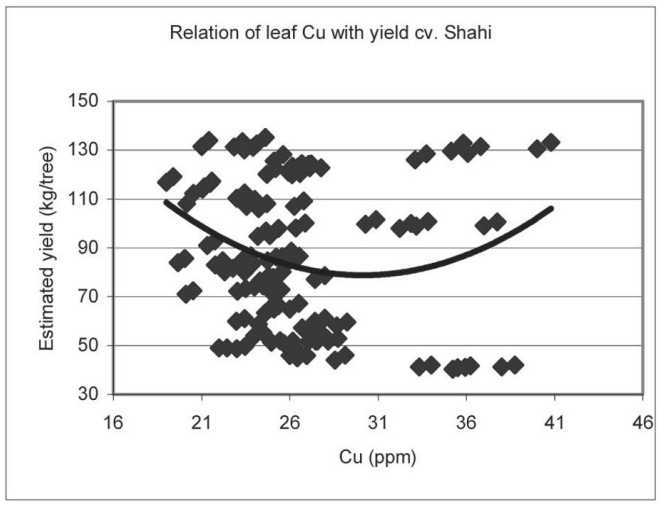


Fig. 1a: Relation of leaf macronutrient content with yield for cultivar Shahi









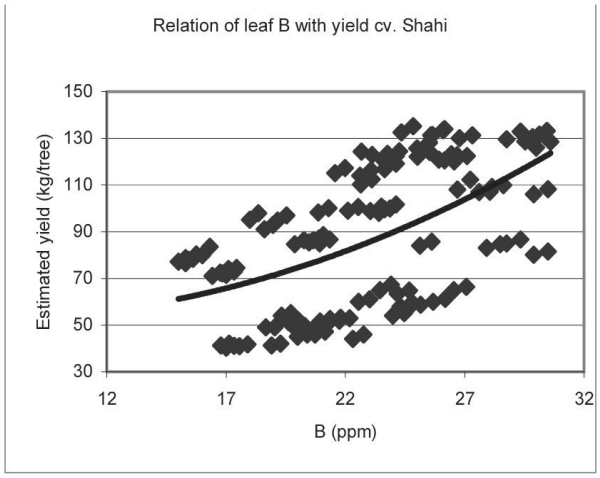
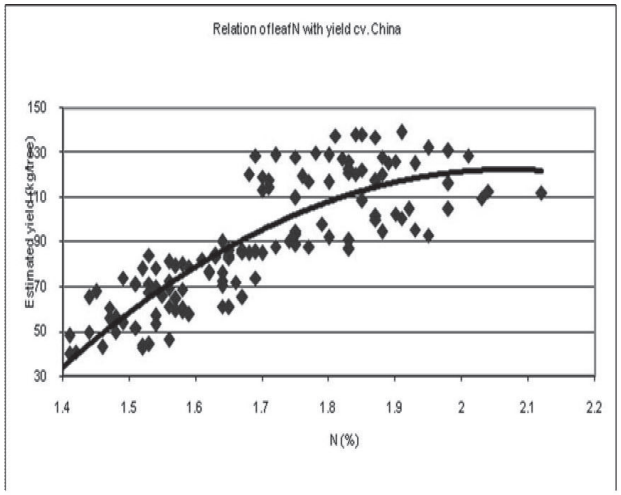
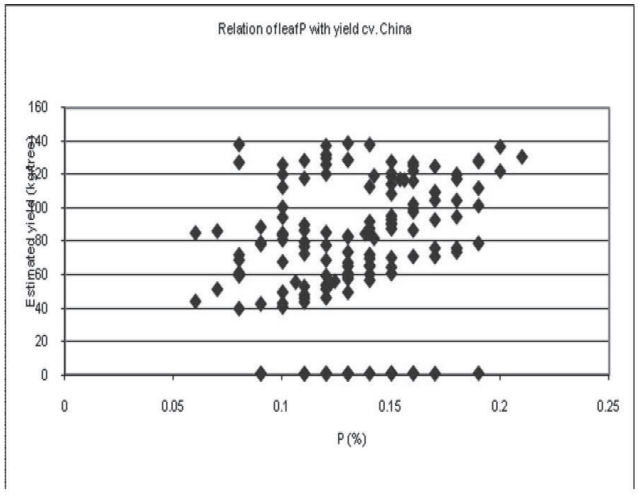
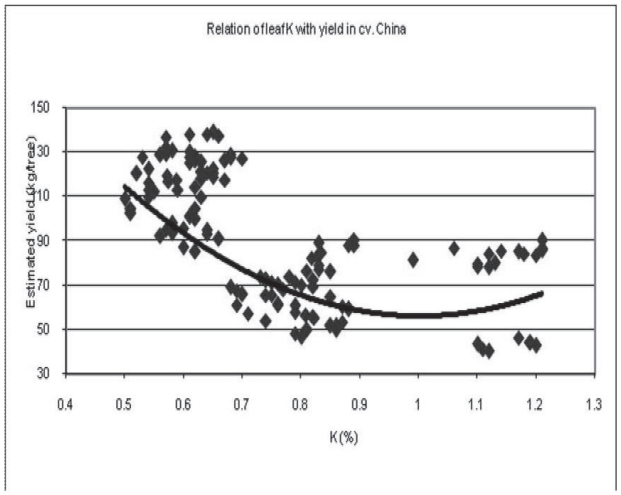
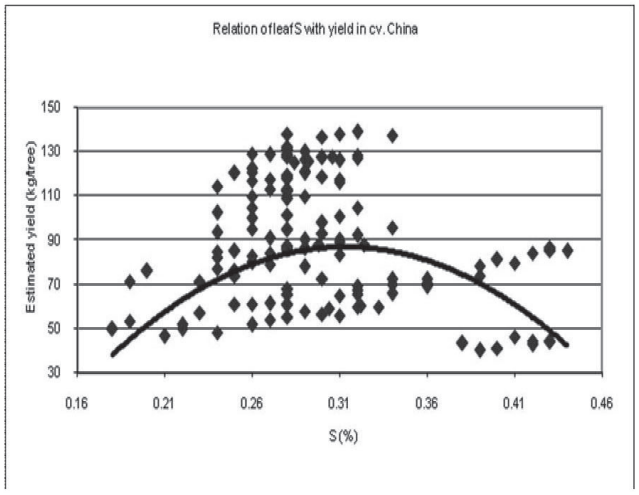


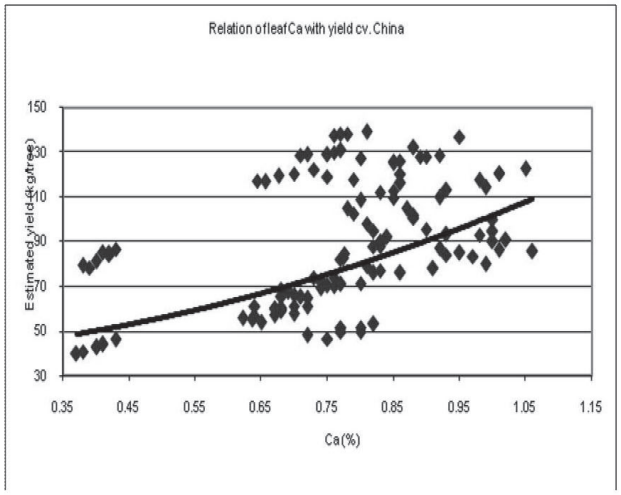
Fig. 1b: Relation of leaf micronutrient content with yield for cultivar Shahi











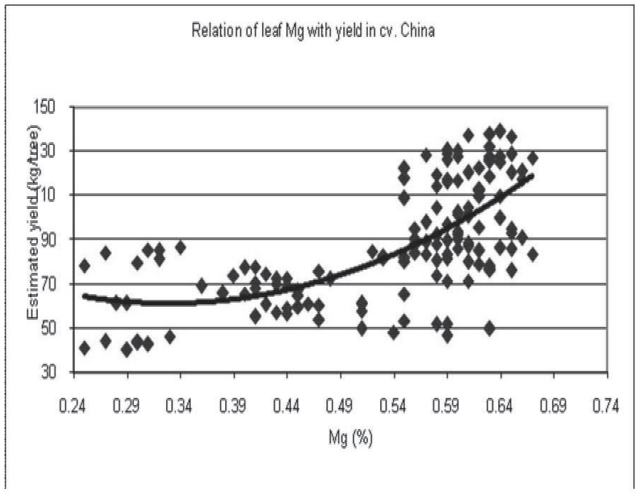
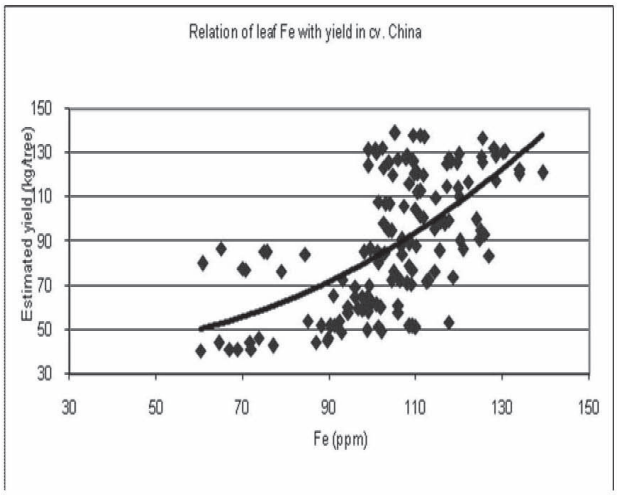
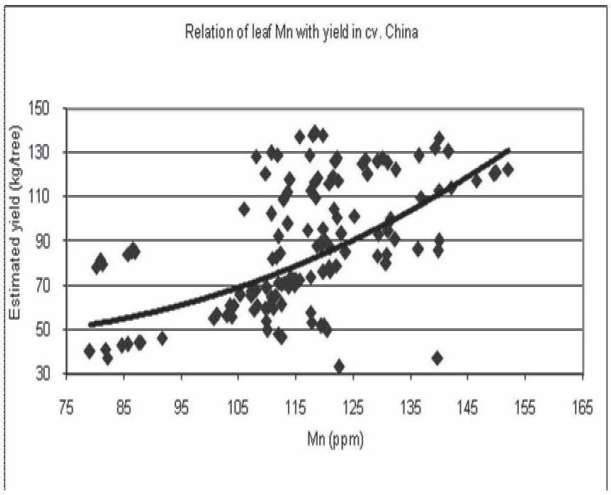
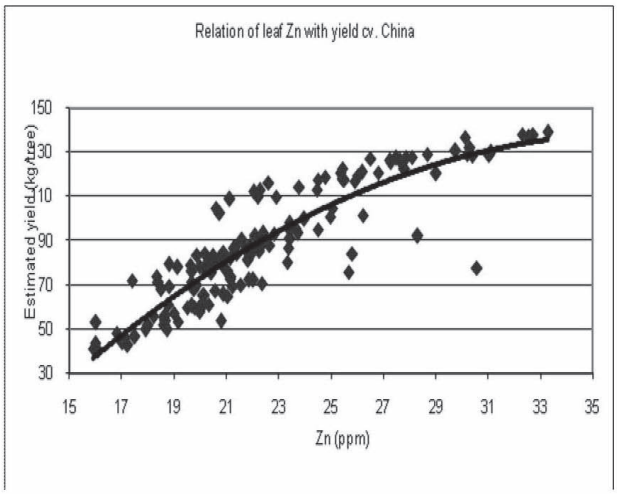
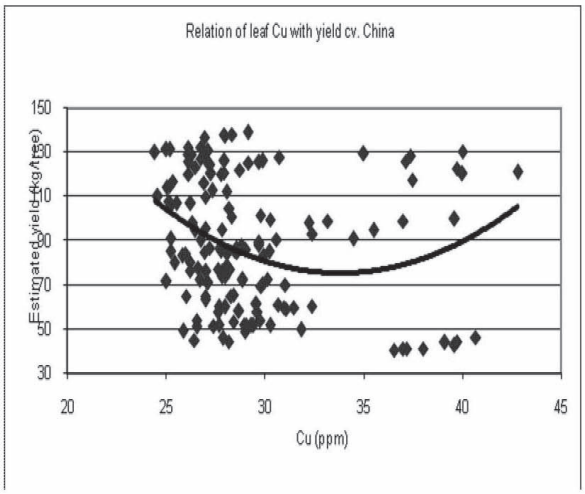


Fig. 2a: Relation of leaf macronutrient content with yield for cultivar China









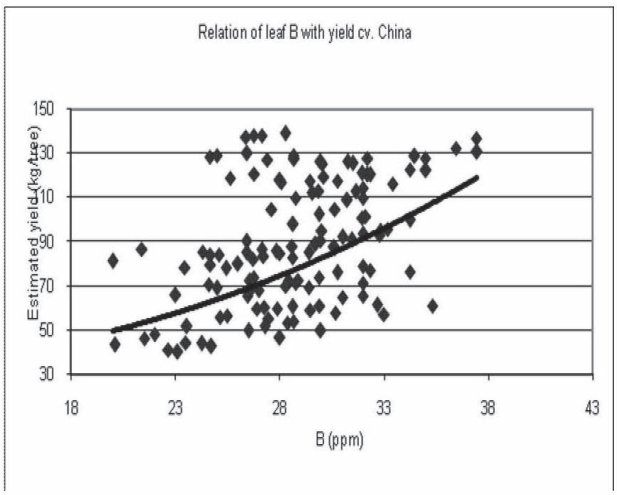


Fig. 2b: Relation of leaf micronutrient content with yield for cultivar China

branches could be sampled (Singh and Chadha, 2009). Shahi and China cultivars could be commercially grown (Singh *et al*.,2010a). Leaf nutrient standard have been developed for the first time in India and would be used as a guide for litchi orchards. Among the nutrients N, P, Ca, Mg, Zn, Fe, B appeared to be more essential, which could be monitored, based on the leaf nutrient content. However, the standard would need refinement based on response to applied fertilizer.

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Ball milled nanosized zeolite loaded with zinc sulfate: A putative slow release Zn fertilizer

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ABSTRACT

Commercially available zeolite was ball milled and the resultant nano sized product was characterized with powder XRD, SEM, FT-IR and BET. Ball milled nano-sized zeolite was mixed with zinc sulfate solution at various concentrations. Zinc sorption and desorption studies were carried out. Nano zeolite loaded with zinc sulfate showed the highest sorption among native and ball milled zeolite and montmorillonite, halloysite and bentonite. The results showed that the highest sorption of 429.5 mg kg^{-1} was observed for nano-zeolite with highest bonding energy (0.33 $mmol^{-1} \times 10^{-3}$) and regression coefficient (0.99). The nano-zeolite recorded 47.9% higher adsorption than nano-montmorillonite. Nano-zeolite showed the release of zinc in three stages. Zn levels decreased sharply in the first 220 hours ($k_1 = 0.53$), and stabilized thereafter. The result also showed that from 300 to 500 hours it follows second stage ($k_2 = 9.44 \times 10^{-3}$). In this stage it supplies 2 ppm of Zn^{2+}. The next stage falls from 500 hours ($k_3 = 1.7 \times 10^{-3}$) and it supplies 1.5 ppm of Zn^{2+} and this condition is static even after 1000 hours. This study suggests that nano-zeolite based Zn fertilizers may be used as a strategy to achieve the slow release of Zn which in turn would improve Zn use efficiency in crops.

Key words: Nano-zeolite, percolation reactor, slow release fertilizer, agriculture, horticulture

INTRODUCTION

Nanotechnology is a highly promising technology that spans many areas of science and technological applications. Rapid advancements in nano sciences and nanotechnologies in recent years have opened up new horizons for agriculture and allied sectors (Adebowale *et al.,* 2005) There are several slow release nitrogenous fertilizers such as neem coated, gypsum coated or tar coated urea to improve the N use efficiency. But the fertilizers vanished at the commercial stage due to the non-availability or practical field problems associated with it. There are no slow release Zn fertilizers available in the market and this is one of the first attempts to look at the slow release pattern of Zn^{2+} as a result of use of nano-carriers such as zeolite.

Zinc is one of the essential micronutrients required for optimum crop growth. Plants take up zinc in its divalent form (Zn^{2+}). Zinc plays an important role in many biochemical reactions within the plants (Ali and Abidin, 2006). Both field crops (maize and sorghum and sugarcane) and horticultural crops (fruits and vegetables) showed reduced photosynthetic carbon metabolism due to zinc deficiency (Almaraz Veronica *et al.*, 2003). Zinc modifies and/or regulates the activity of carbonic anhydrase, an enzyme that regulates the conversion of carbon dioxide to reactive bicarbonate species for fixation to carbohydrates in these plants (Amer *et al*., 2010). Zinc is also a part of several other enzymes such as superoxide dismutase and catalase, which prevents oxidative stress in plant cells (Antonyuk *et al.*, 2008). Zeolites are crystalline alumino-silicate minerals of the alkali and alkaline group elements and have a molecular sieve action due to their open channel network, thereby allowing some ions to pass through while blocking others (Bansiwal *et al.,* 2006, Broadley *et al.,* 2007) All zeolite framework structures have a three dimensional arrangement of TO_4 tetrahedra (T= tetrahedrally co-ordinated atom, usually Si^{4+}, Al^{3+}). These tetrahedra are linked together by the sharing of oxygen atoms and result in an open and stable three dimensional honeycomb structure with an overall negative charge, which is balanced by the cations that move freely in and out of its framework. Ion exchange is an intrinsic property of most zeolites. The cation exchange capacity of a zeolite is a consequence of the degree of substitution of Al^{3+} for Si^{4+} in the framework. These charge balancing cations are generally exchangeable, and channel structure of zeolites is responsible for their function as a "molecular sieve". The channels and cages of a particular zeolite are clearly defined in their dimensions and shape, owing to high crystalline structure of these materials. The open channels thereby allow ions to pass through very freely. On the other hand, it is possible for ions to exchange only partially, because the volumes of the ions are such that these completely fill the intra crystalline space in the channels before 100% exchange is attained

(Chirenje, *et al*., 2005). Thus, zeolite may show great promise as anion and cation carriers for CRFs to control zinc release. It is hypothesized that nano zeolite are negatively charged and they are capable of adsorbing Zn^{2+} ions that facilitate slow steady and regulated release of nutrients. This process will result in nano-fertilizer formulations that assist to regulated release of nutrients and improve zinc use efficiency while preventing environmental hazard.

MATERIALS AND METHODS

Synthesis of zeolite based nano-fertilizer

The zeolite (clinoptilolites) (GM Chemicals, Ahmedabad, India) were of vulcanoclastic origin, with a high cationic interchange capacity [270 C mol (p^+) kg^{-1}]. The clinoptilolite was subjected to ball milling to achieve particles of 30 nm in diameter. Commonly occurring nano-clays such as halloysite, montmorrilionite, and bentonite of 30-40 nm sized particles (Sigma Aldrich chemicals, Bangalore) were used for the study. The physico chemical properties of zeolite and nano-were estimated following standard protocols (Cichocki, 2001).

Particle size analysis

The samples were analyzed for size using DELSA Nano Particle Size Analyzer (Beckman Coulter Counter, Model ZM, Beckman Instruments, Fullerton, CA). All samples were dispersed in deionized water. The sample was sonicated using Sonicator (Model MS-50, Heat Systems, UIltrasonics, Inc., USA) for five minutes at 1500rpm before the average particle size measurement was done.

Shape

The Scanning Electron Microscopy (FEI, Quanta 200, Phillips, Netherlands) was used to get the shape of the nano-clays. About 0.5 to 1.0 g of nano-clay sample was dusted on the carbon conducting tape. Then the tape was mounted on sample stage and the images were taken in 16,000 magnification and 12.50KV.

Surface Area measurement

X-Ray Diffraction technique was used for rapid surface area determination. Approximately one gram of samples was dusted on a glass substrate. Care should be taken that the surface on the glass substrate should be even and it was mounted on the sample stage and diffraction was measured. From this an automatic strip chart record of the sample peak was obtained. The well known Deby-Scherrer formula provides the relationship between mean crystallite size and diffraction line breadth.

Bulk Density

The bulk density, particle density were determined by cylinder method as given by (Gupta and Dhakshinamurthi, 1980). Exactly 20 g of nano-clay samples were taken and transferred to 100 ml measuring cylinder and compacted by tapping 20 times and the volume was noted. Then 50 ml of water was added along the sides of the cylinder using pipette till the entire mass was completely soaked. The cylinder with nano-clays and water kept in an undisturbed condition for at least 30 minutes so that the entire pore space was completely filled with water. The final volume of soil plus water after the expiry of time and the bulk density was calculated.

Colour

Munsell colour chart is used for measuring the colour. The colour of the dried powder of nanoclay was determined by matching the clay samples with the colour chip. The colour chip which closely matches was taken as the colour.

pH and Electrical Conductivity

The pH and Electrical Conductivity (EC) of the nano-clay samples was determined using pH meter and EC meter as per the procedure given by (Cichocki, 2001). Exactly 20g of nano-clay sample was taken into a clean 100 ml beaker.

Table 1: Physical and chemical properties of nano-zeolite and nano-clays

Parameters	Zeolite	Montmorillonite	Halloysite	Bentonite
Physical Properties				
Size (nm)	25-30	30-40	30-40	30-40
Shape	Cubical	Irregular Flakes	Tubular	Round
Surface Area (m^2 g^{-1})	1300	1000	150	80
Bulk Density (g cc^{-1})	0.30	0.46	0.35	0.90
Color	5Y 7/4	5Y 8/1	2.5Y 8/2	2.5Y 7/4
Chemical Properties				
pH of 1% slurry	7.9	8.0	8.2	10.4
EC (dSm^{-1})	0.02	0.02	0.03	0.35
CEC (C mol(p^+) kg^{-1})	270	180	80	60
SiO_2/Al_2O_3 molar ratio	1.95	1.83	1.72	1.65

Then 50 ml of distilled water was added . The content was stirred intermittently and allowed to stand for half an hour. After half an hour the meter reading was recorded.

Cation Exchange Capacity of clays

The Cation Exchange Capacity (CEC) of the clays were determined using the complexes of copper (II) ion with Triethylenetetramine and tetraethylenepentamine (He, e*t al.*, 2002). Two hundred mg of clay sample was added to 35 ml of distilled water. Water was added to make the final volume of 50 ml and transferred into a 100 ml beaker. While stirring the suspension, 10 ml 0.01 M copper(II) trien sulphate solution (1.463 g of triethylenetetramine were dissolved in 100 ml of distilled water) was added. Another solution was prepared by mixing 1.596 g of dry Cu(II) sulphate with distilled water. These solutions were mixed together and added. After 3 min reaction time, the suspension was centrifuged with a micro centrifuge at 13,000 rpm for 3 min. The supernatant solution was carefully removed and the intensity was measured at 620 nm in a 10 mm cuvette against water as a blank. The CEC was then calculated.

Sesquioxide content

Sesquioxide of nano-clay was estimated based on the principle that iron and alumina are precipitated as their respective hydroxides by the addition of ammonia. Then they are converted into oxide forms by ignition and the residue was weighed. First, 50 ml of the HCl extract was pipetted out into a 250 ml beaker. Then 1 g of solid ammonium chloride was added and boiled. The beaker was removed from the flame and diluted ammonium hydroxide was added till the solution becomes alkaline. Again boiled for 4 to 5 minutes and filtered through Whatman No.42 filter paper. The residue was washed with hot water till the filtrate runs free of chloride. The precipitate on the filter paper was dried and transferred to a weighed silica crucible and the constant weight was recorded after ignition. The sesquioxide content was reported in percentage.

Atomic Force Microscopy

Atomic force microscopy (AFM) is a method of measuring surface topography on a scale from angstroms to 100 microns. About 0.1 mg of nano-clays were dissolved in 1ml of acetone. In order to increase the adhesive properties of the mica substrate, poly D-lysine was spread on the surface. One drop of suspension was spread on the mica substrate and setting it aside for few minutes for drying. After drying, the substrate was mounted on sample stage and the images were taken under non tapping mode. The (AFM) images were obtained by means of a NanoScope III (Veeco Dimension 3100 AFM, Veeco Instruments, UK) apparatus using a non tapping mode.

Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is a powerful tool for identifying types of chemical bonds in a molecule by producing an infrared absorption spectrum. Clay sample and IR transparent material like KBr was mixed in the ratio of 2:1 in a mortar and pestle for 30 minutes. Then the mixture was converted into pellets by pressing the prepared mixture with a hydraulic or hand press into a hard disk. The pellet, ideally 0.5 to 1 mm thick was then placed in a transmission holder and scanned. Typically, the pellet technique provides good quality spectra with a wide spectral range and no interfering absorbance bands. Then, the spectra was obtained by Hewlett Packyard Model 8452 A diode Array Spectrophotometer.

Energy dispersive X-ray spectroscopy

Energy dispersive X-ray spectroscopy (EDAX) is an analytical technique used for the elemental analysis or chemical characterization of a sample. (It was done by FEI Quanta 200 EDAX, Phillips, Netherlands).

Surface Modification of nano-clays

Negative charges of the nano-clays were modified by treating them with a surfactant hexadecyltrimethylammonium bromide (HDTMABr) (M/s. Sigma Aldrich, Bangalore) as suggested by (Hernandez *et al.*, 1994). A pre-weighed quantity of nano-clays samples were mixed with HDTMABr solution in a 1:100 (solid: liquid) ratio. The solution was agitated for 7-8 h at 150 rpm in a centrifuge. The solution was then filtered, and the solid residue was washed with double-distilled water and air-dried for 4-6 h. The synthesized surface modified nano-clays (SMNC) were then mechanically ground with a mortar and pestle to a fine particle size.

Zinc sorption

To study the sorption behavior of zinc on surface modified nano-clays, to each 40-ml centrifuge tube, 2.5 g of nano zeolite and 25 ml of 0.5,1.0,1.5,2.0,2.5,3.0,3.5 and 4.0 M zinc sulphate solution were mixed for 24 h at 150 rpm. The mixtures were then centrifuged and the solution was filtered (Whatman no. 42 filter paper pre-washed to eliminate contamination) and zinc concentration was determined by Atomic Adsorption Spectrophotometer (Varian, AA 240, Varian, Inc., USA) The amount of zinc adsorbed was calculated from the difference between the initial and equilibrium solution concentrations. Then the data were fitted with Langumir equations (Jackson, 1973).

Preparation of Slow Release Fertilizers

To each 250ml centrifuge tube, 60 g of nano-clay was

homogenized with 250 ml of 1 M zinc sulphate solution for 8 h and filtered, washed three times with deionized water, and air-dried. The solid:liquid ratio used was 1:10 for the synthesis of zinc loaded nano-clays (Hernandez, *et al.*, 1994).

Soil

The soil samples collected from Maize Research Station, Vagarai, TNAU was used for this study to simulate the actual field conditions.

Experimental Design for Nutrient Release

The chemical reactor designed for a constant flow of (Jakkula, 2005) solution in several studies concerning zeolites was used. Inside the reactor, 5 g of soil overlaid with the nano-zeolite loaded with zinc sulfate were placed. Solutions were collected to determine ammonium and nitrate ions. The mean temperature during the experiment was 25+0.2°C.

RESULTS AND DISCUSSION

Characterization of nano-clays

The nano-zeolite and other nano-clays (montmorillonite, halloysite and bentonite), after loading with Zn^{2+} were scanned through SEM to know the surface morphology (Fig. 1). It showed that the nano zeolite was typical cubic to round in shape. Surface morphology of nano-zeolite after attaching Zn ions (Fig.2) is in confirmation with the results obtained by Kim, *et al.*, 2004.

The data were confirmed by the FTIR. IR spectra of unloaded nano-zeolite having the characteristic peak at 3618, 3440 cm^{-1} (Fig. 4a). The IR pattern of nano zeolite loaded with Zn with the characteristic peaks at wave numbers 1643, 1404, and 840 cm^{-1} (Fig. 4b). There was lot of differences in peaks after loading with Zn. There was shifting in peaks because of loading of Zn. Then it was compared with the reference peak (Fig. 4c). It showed that, the peak at 1503, 1331 and 833 cm^{-1} for zinc in reference graph referred to Zn. The same range of peaks at 1331 and 833 cm^{-1} was also obtained in Fig.1b which was nano-zeolite load with Zn which is in confirmation with the observations made by Langmuir, 1961.

The phase and 3D AFM images of nano-zeolite and nano-clays after loading with zinc are given in the Fig. 3a and 3b. The phase image of zinc loaded nano-zeolite showed the way in which the zinc ions were attached on the surface. Using software of AFM, from a typical horizontal line analysis, for nano-zeolite, we obtained the Roughness average (Ra) to be 1.53 nm, maximum height of the profile above the mean line (Rp) to be 12.56nm, mean of maximum height above mean line (Rpm) to be 7.32 nm, maximum peak to valley height (Rt) to be 18.36 nm and mean of peak to valley height (Rtm) to be 13.19 nm. Similar results were obtained by (Minceva *et al.*, 2007) .

The EDAX images given the mineral composition of nano-zeolite before and after loading Zn. Mineral composition of pure zeolite consists of carbon (11.74%), oxygen (44.79%), magnesium (8.18%), aluminum (4.66%), silica (26.19%) and calcium (4.43%) (Fig. 5). The result showed that, the zinc ions was adsorbed by the zeolite and it was

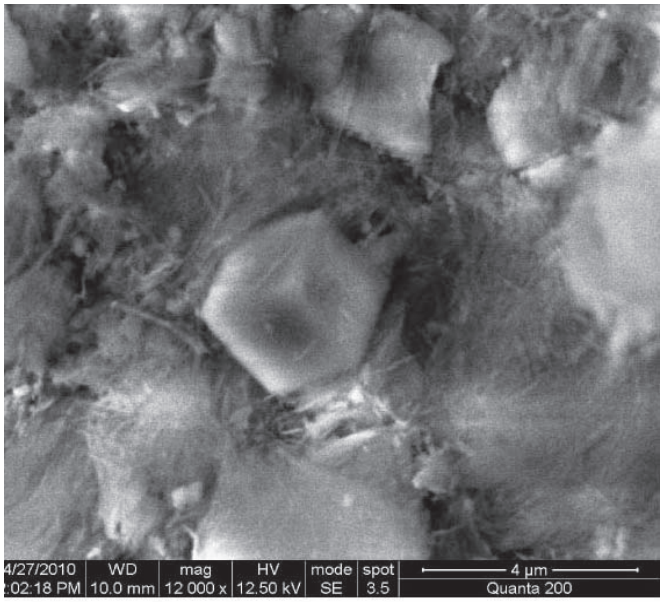


Fig. 1: Nano zeolite

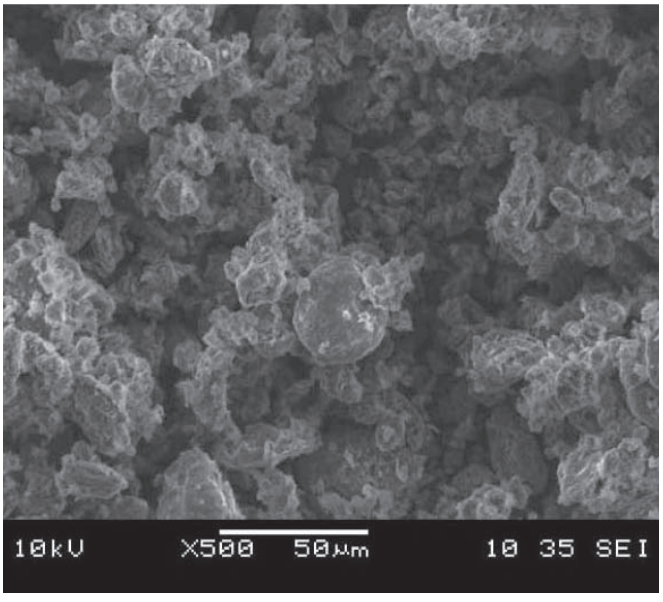
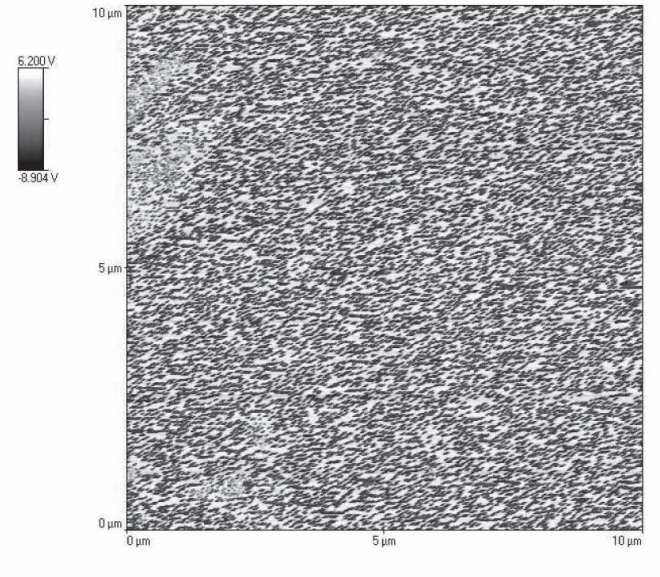


Fig. 2: Nano zeolite after loading zn



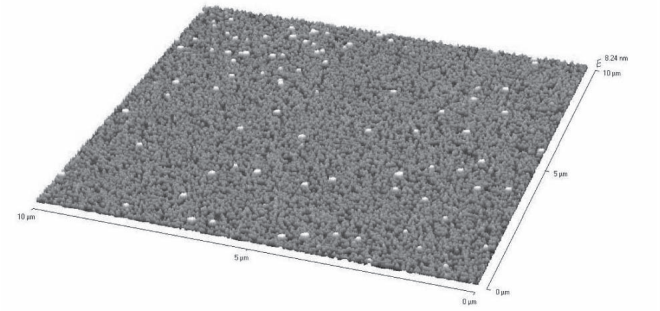


Fig. 3a: Phase image of zinc ions on zeolite
Fig. 3b: 3D view of zinc ions on zeolite

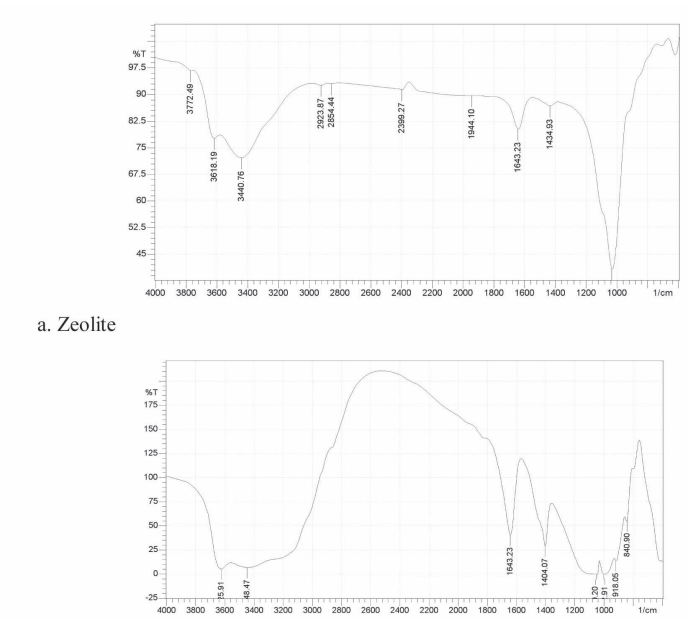
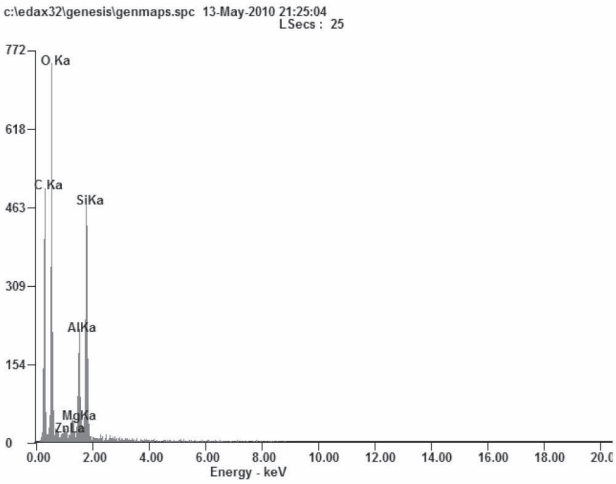


Fig. 4: FT-IR spectra of zeolite, zeolite loaded with zinc and the reference



Element	*Wt%*	*At%*
CK	45.39	57.62
OK	30.71	29.27
ZnL	00.38	00.09
MgK	01.20	00.75
AlK	06.68	03.77
SiK	15.64	08.49
Matrix	Correction	ZAF

Fig. 5: EDAX image of nano-zeolite loaded with Zn

included in the mineral composition as 0.38%. Similar results were obtained by several researchers (Ming and Allen 2006, Murat *et al*., 2006, Patrick and Francoiz, 2008).

Zinc Sorption

The zeolite and nano-clays montmorillonite, halloysite and bentonite were loaded with zinc sulphate of different molar concentrations (Fig. 6). The zinc sorption on nano-zeolite and nano-clays showed that the amount of zinc sorbed increases with the increase in equilibrium Zn^{2+} concentration (Murat *et al.,* 2006). This increase continues up to 4500 ppm and beyond this value, there is not a significant change at the amount of adsorbed metal ions (Patrick and Francosis, 2008). This plateau represents saturation of the active sites available on the zeolite samples for interaction with metal ions (Peric and Medvidovic, 2004). It can be concluded that the amount of metal ions adsorbed into unit mass of the zeolite at equilibrium (the adsorption capacity) rapidly increases at the low initial metal ions concentration and then it begins to a slight increase with increasing metal concentration (Sheta *et al.,* 2003). These results indicated that energetically less favorable sites become involved with increasing metal concentrations. Among them, zeolite adsorbed more zinc (220ppm) than other nano-clays, which was followed by nano-montmorillonite (175 ppm). The adsorption rate obtained with the zeolite seemed to be very satisfactory and clinoptilolite can be accepted as an efficient adsorbent for zinc removal (Subramanian and Tarafdar, 2011).The other clays nano-halloysite and nano-bentonite more or less similar amount of Zn^{2+} from the solution. Modification of clay minerals for the adsorption of Zn^{2+} has been reported by many researchers (Tiwari *et al.*, 2008, Unuabonah *et al.*, 2007, Welch,1995).

The experimental data fitted well with the linearized form of Freundlich models (Table.2). The applicability of isotherm model to the Zn-clinoptilolite system implies that both monolayer adsorption and heterogeneous surface conditions exist. The adsorption of Zn^{2+} ions on the clinoptilolite is thus complex, involving more than one mechanism. Freundlich isotherm assumes heterogeneous surface with a non-uniform distribution of heat of adsorption. The result showed that the highest sorption of 429.5 mg kg^{-1} was observed for nano-zeolite with highest bonding energy (0.33 $mmol^{-1}x\ 10^{-3}$) and regression coefficient (0.99). The nano-zeolite recorded 47.9% higher adsorption than nano-montmorillonite. The nano-montmorillonite showed the adsorption of 223.6 mg kg^{-1} with the bonding energy of 0.29 $mmol^{-1} \times 10^{-3,}$ followed by nano-halloysite 147.3mg kg^{-1} and nano-bentonite 93.5 mg kg^{-1}. Among them, nano-zeolite adsorbed higher amount of zinc than all other nano-clays. Thus, the Langmuir isotherm provided the best correlation for modified clinoptilolite. Zeolite, which is known as the best absorbent material for heavy metals, has the highest CEC and shows high adsorption rates (Yeshchenko, *et al*., 2005).

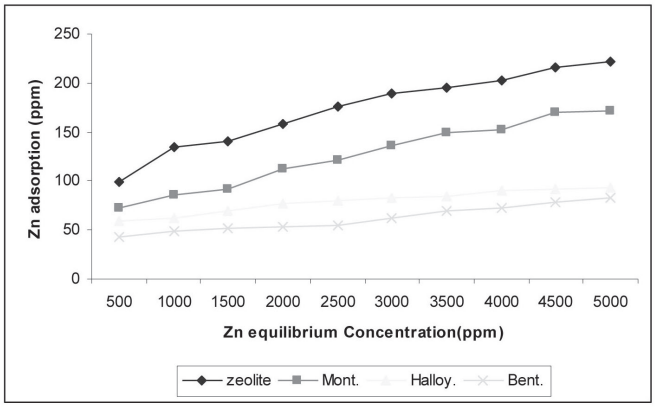


Fig. 6: Sorption zinc on zeolite and nano-clays (montmorillonite, halloysite and bentonite) under varying molar concentration of zinc sulphate

Table 2. Sorption Maxima (b), Bonding Energy (K), and Coefficients of Regression for Zn sorption on NC (r^2)

Nanoclays	b (mg/kg)	K ($mmol^{-1}x\ 10^{-3}$)	r^2
Nano zeolite	429.5	0.33	0.99
Nano montmorillonite	223.6	0.29	0.97
Nano Halloysite	147.3	0.24	0.96
Nano Bentonite	93..5	0.20	0.94

Slow Release of Zinc

At the start of the experiment (Fig.7), a maximum concentration of 22 ppm Zn was observed in the leachate from nano-zeolite followed by nano- montmorillonite (12 ppm), nano-halloysite (5 ppm), nano-bentonite (3 ppm) and zinc sulphate (2.5 ppm). Furthermore, it can be seen that in the initial stage, Zn^{2+} release from all the nano-clays occured rapidly and attained a static at about 312 h, after which slow release was observed. The data revealed that the entire available Zn from zinc sulphate was exhausted after 120 h beyond which the concentration of Zn^{2+} reached below detectable limits. However, the release of zinc from nano-zeolite was continued even after 1176 h, with a concentration of 5 ppm. The same results were reported by several researchers (Zhou and Huang, 2007). It was reported that, the mechanism for this effect may be sparingly soluble minerals are dissolved by the sequestering effect of the exchanger, thereby releasing trace nutrients to zeolite exchange sites where they are more readily available for uptake by plants. Zeolites can become an excellent plant growth medium for supplying plant roots with additional vital nutrient cations and anions. The nutrients are provided in a slow-release, plant root demand-driven fashion through

the process of dissolution and ion exchange reactions. The absorption of nutrients from the soil solution by plant roots drives the dissolution and ion exchange reactions, pulling away nutrients as needed. The zeolite is then "recharged" by the addition of more dissolved nutrients. In effect, zeolites increase nutrient retention, reduce environmental nutrient losses and reduce fertilizer requirements by establishing a replenishable and balanced nutrient supply in the plant root zone.

Nano-zeolite showed leaching of zinc in three stages

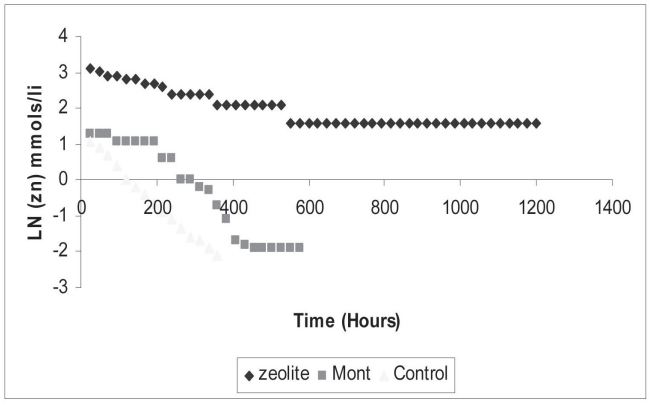


Fig. 7: Kinetics of zinc release from nano-zeolite, nano-clays and zinc sulphate

(Fig. 7). Zn levels decrease sharply in the first 220 hours ($k_1 = 0.53$), and stabilize thereafter. The result also showed that from 300 to 500 hours it follows second stage ($k_2 = 9.44 \times 10^{-3}$). In this stage it supplies 2 ppm of zinc. The next stage falls from 500 hours ($k_3 = 1.7 \times 10^{-3}$) and it supplies 1.5 ppm of zinc and this condition is static even after 1000 hours. This may be due to the fact that, the zeolite not only increases nutrient retention but also achieves the slow release process of nutrient for gradually releasing the nutrient to the plant so as to reduce environmental nutrient losses of the soil by means of the ion exchange ability. Therefore, the natural environmental materials of the diatomite and the zeolite are mixed to be the plant growth medium for agriculture to increase crop yields.

The data suggest that the nano-zeolite based fertilizers supply Zn for an extended period of up to 40 days while such release ceased to exist within 10-12 days. The trend of Zn release pattern can be exploited in the development of nano-fertilizer that ensures sustainable plant development besides environmental safety. More research is needed to address the field response of nano-formulations and biosafety issues concerning nano-fertilizers.

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Aonla pomace as substrate for endoglucanase production using *Trichoderma harzianum*

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ABSTRACT

Submerged fermentative (SmF) production of endoglucanase by a cellulolytic fungus *Trichoderma harzianum* any isolet number using aonla (*Emblica officinalis* Gaertn.) pomace as a substrate was standardized. Optimum production of the enzyme (0.57 ± 0.05 U/ml) was obtained at pH 4.0 and 30 ºC temperature using substrate concentration of 1.5% (w/v) supplemented with ammonium sulphate (1.5% w/w of substrate) as nitrogen source on 3rd day of fermentation. The soluble extracellular protein was extracted and partially purified by acetone to an extent of 2.395 fold. Protein profiling on 12% Sodium dodecylsulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) revealed three bands with molecular size corresponding to 86, 68 and 46 kDa, suggesting the isomeric forms of endoglucanase. The optimum endoglucanase activity in partially purified sample was achieved at pH 6.0 and 30 ºC temperature. The Michaelis-Menten constant (K_M) and V_{max} were found to be 13.89 mg/ml, and 0.215 µmole/min/mg, respectively.

Key words: Aonla, endoglucanase, submerged fermentation, *Trichoderma harzianum.*

INTRODUCTION

Gooseberry (*Emblica officinalis* Gaertn.), commonly called as aonla or amla, is one of the important indigenous fruits of the Indian subcontinent. Various products are prepared from aonla, which include juice, squash, syrup, jam, candy, shreds, sauce, etc. In recent years, its juice is gaining popularity and commercial significance owing to its nutraceutical and pharmaceutical properties (Tambekar and Dahikar, 2010). During aonla juice extraction voluminous amount (upto 35%) of solid pomace is generated, which is a rich source of ligno-cellulosic fibre (23.5% w/v). Endoglucanase (also known as endo-β-1-4 glucanase;1, 4-β glucano hydrolase; EC 3.2.1.4) is involved in the break down of inner cellulose chain. It is a major constituent of cellulase enzyme complex, which is widely used in chemical, fuel, food, animal feed, brewery, wine, textile, laundry, pulp, paper and other agro-based industries. There are reports of endoglucanase production using sugar cane, rice straw and citrus processing waste (Tao *et al.,* 2011). The present paper reports endoglucanase production using aonla pomace as substrate. The conditions for submerged fermentative production of endoglucanase by *Trichoderma harzianum*, a potent cellulolytic fungus, were standardized. The enzyme was also partially purified and characterized and the enzyme kinetics was worked out.

MATERIALS AND METHODS

Substrate

Aonla pomace (dried and powdered) was used as substrate for endoglucanase production.

General fermentation protocol

The production medium (100 ml in triplicate) was autoclaved and inoculated with *Trichoderma harzianum* isolet number and (10^5 spores). The flasks were incubated at 30°C±1°C under stationary conditions and sampling was done at specific time intervals of 5 days unless other wise stated.

Protein quantification

The protein concentration of crude as well as partially purified enzyme was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

Enzyme assay

The reaction mixture, containing 0.4 ml of 2% carboxymethyl cellulose sodium salt (Hi-media, India) in 0.05M acetate buffer (pH 5.0) and 0.1 ml of the enzyme solution, was incubated at 30 ºC for 60 min. Endoglucanase activity was quantified by measuring the amount of reducing sugars released in the medium (Vintila *et al.,* 2010). One

unit of endoglucanase is the amount of enzyme that produced one µ mole of glucose min^{-1} ml^{-1} under assay conditions.

Optimization of pH and fermentation duration

The pH of production medium was adjusted to 2.5, 4.0, 5.5, 6.5 and 8.5. The specific pH level for production of maximum enzyme was recorded. Fermentation period (1-5 day) was also standardized for optimum endoglucanase production.

Optimization of substrate concentrations

Substrate concentrations (0.5-2% w/v with increments of 0.5%) and nitrogen addition (in the form of ammonium sulphate @ 0.5-2% w/w of substrate with increments of 0.5%) were optimized.

Effect of inorganic salts addition on endoglucanase production

Inorganic salts viz. calcium as $CaCl_2$. $2H_2O$ (4.5, 9.0 and 45 mM), iron as $FeCl_3$ (4.5, 9.0, 45 mM), magnesium as $MgCl_2.7H_2O$ (0.33, 3.3 and 33 mM) and Zinc as $ZnSO_4$ (1.75, 17.5 and 35.5mM), were added individually into the 100 ml production medium. The endoglucanase activity was determined in the culture filtrate.

Enzyme purification

Culture filtrate to acetone ratio was standardized for optimum enzyme purification. The culture filtrate containing extracellular protein was partially purified using culture filtrate to acetone ratio, i.e., 1:1, 1:2, 1:3 and 1:4 at 4 °C for 24 h. The precipitate was collected by centrifugation at 12,000 g, at 4 °C for 20 min and re dissolved in10 ml of 0.05 M acetate buffer of pH 5.0.

Enzyme characterization

Optimization of pH and temperature for maximum enzyme activity

The effect of pH (3.0 to 7.0 in the increments of 1unit) on endoglucanase activity was studied using citrate/phosphate buffer (0.02 M) as described by Ali *et al.,* (2008). For temperature optimization studies, the enzyme extract was incubated at incremental temperatures of 10 ºC from 20-60 ºC for 30 min and then assayed for endoglucanase activity.

Sodium dodecylsulfate

Polyacrylamide gel (SDS-PAGE) electrophoresis: Electrophoretic analysis of purified protein was done under alkaline conditions at pH 8.8 on 10% native polyacrylamide gel and 12% denaturating PAGE (SDS-PAGE) as described by Laemmli and Favre (1973). Electrophoresis was performed at constant voltage of 100V for 4 h in Vertical Mini Gel apparatus (Bangalore Genei, India). After electrophoresis, the gels were stained with glycoprotein staining using Schiff's reagent. The molecular size of the enzyme (kDa) was determined from the gel based standard protein markers [(β-galactosidase (110 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (30 kDa) and bamHI-25 (25 kDa)] coelectrophoresed with the protein samples by relative front (R_f) value determination based on migration distance.

Determination of kinetic constant

Kinetics of the enzyme was analyzed using Lineweaver-Burke and Michaelis-Menten plots (Sanghi *et al.,* 2010). The enzyme kinetics experiment was performed by measuring the initial enzyme reaction velocity at different substrate concentrations of carboxy methyl cellulose sodium salt in 50mM acetate buffer (pH 5.0). The Lineweaver-Burke plot was used to establish the Michaelis constant (K_M) and maximum velocity (V_{max}) of the enzyme reaction. In all experiments three replications were taken and mean values were calculated.

RESULTS AND DISCUSSION

Optimization of fermentation temperature and pH for enzyme production

Temperature and pH are the most important factors, as they markedly influence enzyme activity. Maximum endoglucanase activity (0.024 ± 0.004U/ml) was recorded at 30 ºC while the reducing sugar content decreased (Table 1).

Table 1: Optimization of fermentation temperature for endoglucanase production

Temperature(ºC)	Endoglucanase[a](U/ml)	Protein[b](mg/ml)	Reducing sugars [c](mg/ml)
20	0.018±0.001	5.014±0.62	3.386±0.09
30	0.024±0.004	4.161±0.63	3.085±0.26
40	0.010±0.001	7.090±0.84	4.830±0.37

[a]One unit of endoglucanase is the amount of enzyme that produced one µmol of glucose min^{-1} ml^{-1} under assay conditions
[b]Quantification of protein in culture filtrate (enzyme) ± standard deviation
[c]Quantification of reducing sugars present in culture filtrate (enzyme) ± standard deviation

Table 2: Optimization of fermentation pH for endoglucanase production

pH	Endoglucanase[a](U/ml)	Protein[b](mg/ml)	Reducing sugars [c](mg/ml)
2.5	0.003 ± 0.001	2.287 ± 0.15	6.012 ± 0.32
4.0	0.087 ± 0.01	2.430 ± 0.62	6.854 ± 0.43
5.5	0.047 ± 0.01	2.652 ± 0.30	5.331 ± 0.54
6.5	0.002 ± 0.01	2.738 ± 0.21	2.631 ± 0.24
8.5	0.000 ± 0.001	2.477 ± 0.45	0.831 ± 0.06

[a]One unit of endoglucanase is the amount of enzyme that produced one ìmol of glucose min^{-1} ml^{-1} under assay conditions
[b]Quantification of protein in culture filtrate (enzyme) ± standard deviation
[c]Quantification of reducing sugars present in culture filtrate (enzyme) ± stamdard devoatopm

Later might have triggered enzyme production since the specific activity was highest (0.006 U/mg proteins). Further increase in temperature resulted in decrease of the activity of the enzyme. Sun *et al.* (2010) have also reported maximum endoglucanase production at 30 ± 2 ºC by *Trichoderma sp*. using apple pomace as substrate. Since enzymes are secondary metabolites produced during exponential growth phase, incubation at high temperature leads to poor growth which eventually reduces the enzyme yield (Sabu *et al.*, 2002). Maximum enzyme production (0.087 ± 0.01U/ml) and specific activity (0.036 U/mg protein) was observed at pH 4.0 (Table 2).

Substrate concentration for enzyme production

The maximum enzyme activity (0.15±0.01U/ml) was observed at 1.5 % aonla pomace. Higher pomace concentration (> 1.5 % w/v) in the production medium produced negative effect and the enzyme yield was reduced considerably (Fig 1), which might be due to the feed back inhibition. Rashid *et al.* (2009) also reported that 1.5% substrate concentration (oil palm waste) was optimum for cellulase production.

Nitrogen source for enzyme production

Nitrogen addition as ammonium sulphate had shown positive effect on endoglucanase production. Among various concentrations of ammonium sulphate tested, a concentration of 1.5 % (w/w) resulted in maximum enzyme (0.346 ± 0.02 U/ml) production (Fig 2). Any increase or decrease in the nitrogen concentration resulted in declined enzyme activity. Sun *et al.*, (2010) reported 14% increase in cellulase production by *Trichoderma sp*. on apple pomace supplemented with 1.0% ammonium sulphate.

Optimization of fermentation time for enzyme production

Optimum enzyme production (0.57 ± 0.05 U/ml) was observed on the third day of incubation (Fig 3), when protein concentration was maximum (2.3 ± 0.23mg/ml) and

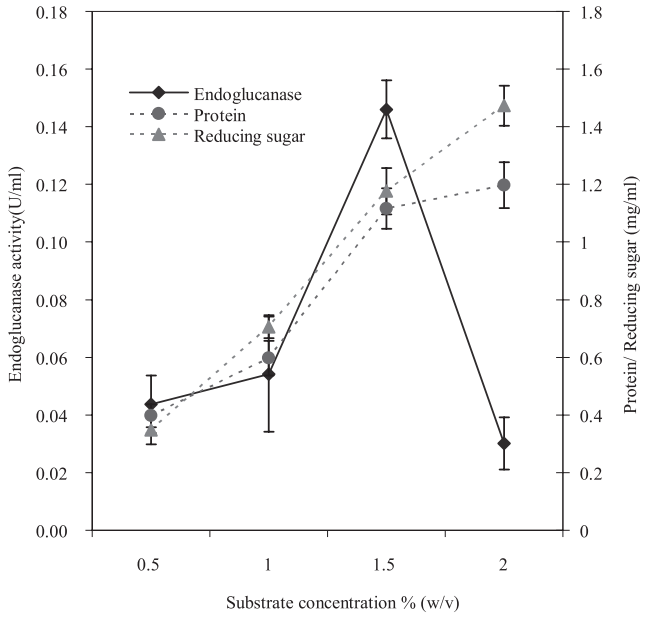

Fig. 1: Effect of substrate (aonla pomace) concentration on the endoglucanase production using *T. harzianum.*

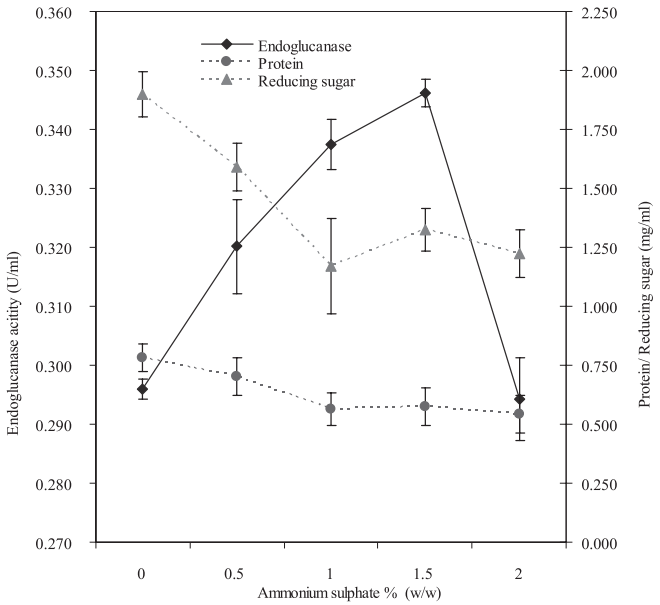

Fig. 2: Effect of ammonium sulphate addition on the endoglucanase production

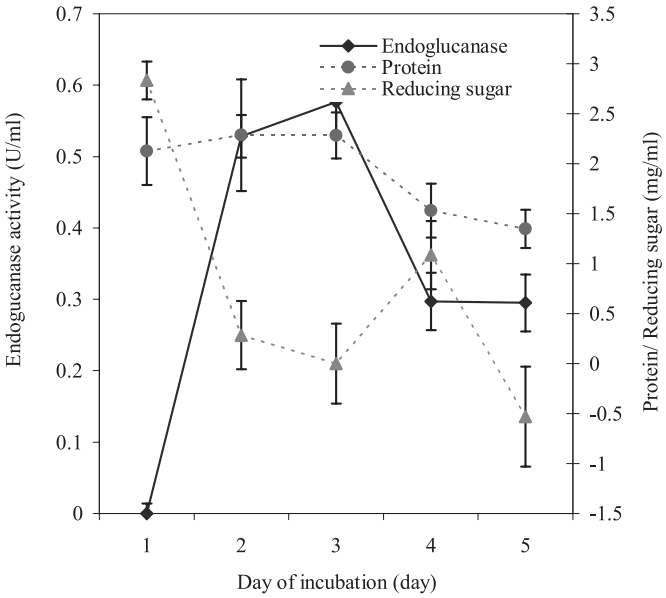

Fig. 3: Effect of incubation period on endoglucanase production under optimized conditions.

reducing sugars were low (0.011 ± 0.004). The reduction in the reducing sugars might have created a stress for carbon source and hence, activated the secondary carbon source (aonla fibre) utilization resulting into increased endoglucanase production. Odeniyi *et al.,* (2009) reported optimum fermentation on third day of incubation using palm fruit industrial residue as carbon source. However, Khan *et al.* (2007) and Ali *et al.* (2008) reported highest endoglucanase activity on fourth day of fermentation by *Trichoderma harzianum* and *Phanerochaete chrysosporum*, respectively in synthetic CMC medium.

Effect of inorganic salt addition for enzyme production

Inorganic salts are nutrients needed in small quantities for enzyme production. However, the results indicated the inhibitory effect of inorganic salts (Ca^{+2}, Mg^{+2}, Zn^{+2} and Fe^{+3}), addition on the enzyme production. Addition of Mg^{+2} (0.33 mM), Ca^{+2} (4.5 mM), Fe^{+3} (4.5 mM), and Zn^{+2} (1.75 mM) to growth medium, decreased the enzyme production from 0.88 (control) to 0.44, 0.7, 0.2, 0.3 U/ml respectively. Increasing the inorganic salt concentration (as indicated in material and method) inversely affected the enzyme production. Rashid *et al.* (2009) reported negative effect of Mg^{2+} on endoglucanase production.

Enzyme purification

Acetone precipitation has been reported as an efficient method for concentrating and desalting sample for protein purification (Jiang *et al.,* 2004). In the present study the concentration of acetone for optimum protein purification was standardized. Maximum purification of extracellular protein could be achieved when present filtrate was precipitated with acetone in 1:3 ratio resulting into 2.39 fold purification at 9.58% protein concentration. The

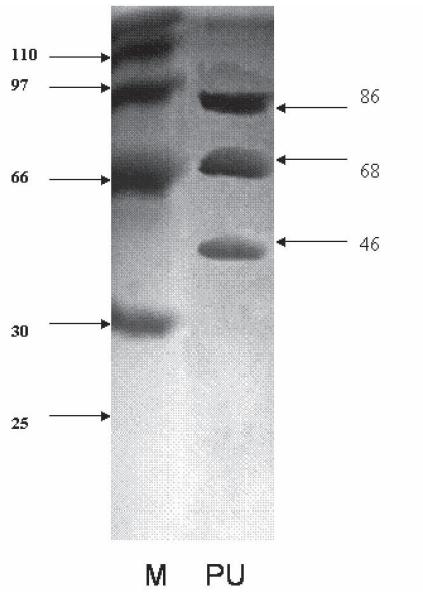

Fig. 4: SDS-PAGE profile of crude protein and purified endoglucanase.
Abbreviations: M-Marker (β-Galactosidase (110kDa), Phosphorylase (97kDa), Bovine Serum albumin (66kDa), Carbonic anhydrase (30kDa), BamH I-25 (25kDa))
PU-Purified endoglucanase: 86, 68 and 46 kDa

Table 3: Purification profile of endoglucanase produced from aonla pomace by *Trichoderma harzianum as* achieved in different acetone concentrations.

Purification step	Volume (ml)	Total Endoglucanase (*U)	Total Protein (mg)	Specificactivity (U/mg)	Yield (%)	Purification (fold)
Crude culture filtrate	200	319.44	345.67	0.92	100.00	1
Supernatant	190	74.22	164.93	0.004	23.23	0.01
Partial purification by acetone (1:1)	10	9.04	11.54	0.783	2.83	0.851
Partial purification by acetone (1:2)	10	8.04	15.54	0.517	2.51	0.56
Partial purification by acetone (1:3)	10	30.6	13.83	2.210	9.58	2.39
Partial purification by acetone (1:4)	10	9.04	25.82	0.350	2.83	0.37

*One unit of endoglucanase is the amount of enzyme that produced one ìmol of glucose min^{-1} ml^{-1} under assay condition

acetone precipitation at 1:4 ratio resulted in only 0.379 fold purification at 2.83% protein concentration (Table 3).

Molecular size determination by SDS-PAGE

Protein profiling on 12% SDS-PAGE revealed three bands corresponding to 86, 68 and 46 kDa molecular sizes (Fig 4), suggesting the isomeric forms of endoglucanse, while it was observed as single band on 10% Native gel. Since cellulase is a heteromeric multienzyme complex, these three bands might correspond to different components of cellulase enzyme complex in *Trichoderma harzianum*. Kim *et al.*, (1994) reported average molecular size of 51, 59 and 41 kDa for endoglucanase in *Trichoderma viride*. Saravanan *et al.* (2007) reported 52-62 kDa sized endoglucanase in *Trichoderma reesei*. On an average, molecular size of microbial endoglucanase has been reported to be in the range of 50-94 kDa (Chen *et al.*, 2004; Ariffin *et al.*, 2006; Aygan and Arikan, 2008).

Optimization of pH, temperature and substrate concentration for endoglucanase activity

The optimum endoglucanase activity (0.116U/ml) was achieved at pH 6. Lower activity of 0.106 U/ml and 0.08U/ml were observed at pH 5 and 7, respectively. Ali *et al.*, (2008) reported pH 5 as ideal for endoglucanase activity. A temperature of 30ºC was found optimum for maximum endoglucanase activity (0.25 U/ml). Lower activities viz. 0.18 U/ml and 0.21 U/ml were observed at 20 ºC and 40 ºC, respectively. Most of the carboxymethyl cellulose degrading endoglucanases are known to exhibit maximum activity in the temperature range of 30-60 ºC (Ariffin *et al.*, 2006; Ali *et al.*, 2008).

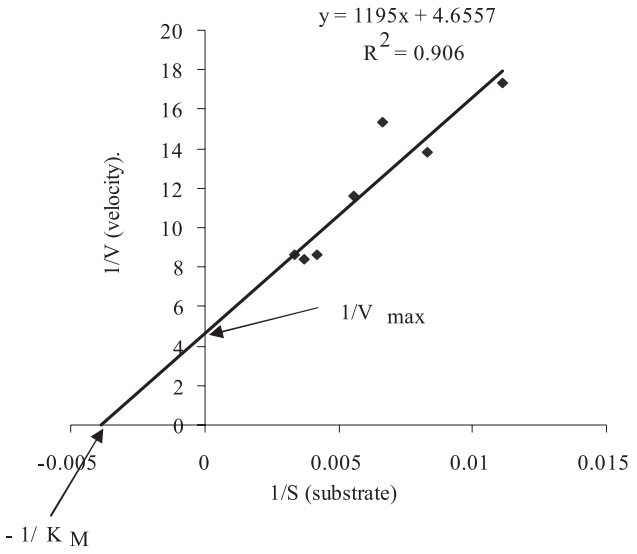

Fig. 5: Lineweaver-Burke plot showing the Michaelis–Menten-type kinetics of endoglucanase on the carboxylmethyl cellulose under the standard assay condition specified, indicating the K_M value under maximum velocity (V_{max})

Enzyme kinetics

The effect of the substrate concentration on the enzyme activity indicated that endoglucanase activity increased with the increase in carboxymethyl cellulose concentration up to 24 mg/ml and decreased thereafter. The Michaelis constant (K_M) and V_{max} values were found to be 13.89 mg/ml and 0.215 µmole glucose released sec^{-1} mg^{-1} protein, respectively. Using Line weaver-Burke plot (Fig 5) the R^2 value was worked out to be higher than 0.9 which depicted Michaelis–Menten-type of kinetics for endoglucanase on the carboxymethyl cellulose under the standard assay conditions. De Castro *et al.* (2010) reported K_M (19.39 mg/ml) and V_{max} (0.0948 m.mole/L) for endoglucanase production using *Trichoderma harzianum* on pure carboxymethyl cellulose. The lower K_M values observed in our study might be due to aonla pomace as substrate.

The study indicated that aonla pomace may serve as suitable substrate for endoglucanase production. More and more emphasis is now being laid on utilization of fruit processing industry waste for value added products. In this context, the present research may find useful application.

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Genetic diversity and phylogenetic relationship among small cardamom (*Elettaria cardamomum* Maton.) cultivars and related genera using DNA markers

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ABSTRACT

Elettaria cardamomum Maton is a monotypic genus native to India but a few related tribes like *Amomum, Aframomum, Alpinia* and *Hedychium,* coexist with small cardamom. In the present study, about 11 species representing 5 major tribes viz., *Amomum, Aframomum, Alpinia, Hedychium* and *Elettaria* and 96 collections of cardamom germplasm representing the range of genetic variability available in different Cardamom Research Centers were studied using RAPD, PCR - RFLP and ISSR polymorphism. The study indicated that all the genotypes are distinct from each other and there are no duplicates in germplasm collections studied. Among the released varieties and promising genotypes, the lines developed by different research centers by selection, clustered together, indicating narrow range of diversity from which these selections are made. But the distinct grouping of hybrids showed that the varieties developed by hybridization have higher divergence. Hence, hybridization rather than selection should be the preferred method of improvement to broaden the genetic base of cultivated cardamom. Among the eleven species representing 5 major tribes studied, *Amomum* is closer to cultivated cardamom. The present study revealed that the genetic variation expressed by molecular markers is relatively low among the cultivated cardamom though most of them are clear morphological variants and this is expected from a monotypic genus like *Elettaria*.

Key words: Zingiberaceae, Cardamom, India, monotypic genus, ISSR, RAPD, PCR- RFLP

INTRODUCTION

India is the centre of diversity of small cardamom (*Elettaria cardamomum* Maton.), which is one of the most important economical spices, along with saffron and vanilla. Cardamom var. *minor* acclaimed, as the 'Queen of spices', is the true cardamom belonging to the family Zingiberaceae under natural order Scitaminae. The genus *Elettaria* consists of about six to seven species distributed in India, Sri Lanka, Malaysia and Indonesia. Of these only *E. cardamomum* is economically important (Hooker, 1894, Gamble, 1925, Holttum, 1950; Mabberly, 1987; Madhusoodanan *et al*., 2002). In India *Elettaria* is a monotypic genus represented with by only *E. cardamomum.* Two varieties are recognized based on the size of the fruits. They are *Elettaria cardamom* var. major, with larger fruits and *Elettaria cardamom* var. minor comprises of all the cultivated races (Purseglove *et al.,* 1981). Cardamom substitutes, which sometimes appear in trade and are given the name cardamom, are obtained from *Aframomum* spp. in Africa and *Amomum* spp. in Asia. Small cardamom occurs wild in the gaps of evergreen mountain monsoon forests of Western Ghats in South India and in the western high lands of Sri Lanka (Wardini and Thomas, 1999). The other main cardamom cultivating countries are Guatemala, Srilanka, Thailand and Sumatra (Purseglove *et al.* 1981). The total area under the crop in India during 2009-10 is 90,000 ha which spreads over Kerala, Karnataka and Tamilnadu (Directorate of Economics and Statistics, Ministry of Agriculture 2011, New Delhi). *E. cardamomum,* generally propagated by seeds and suckers, exhibits considerable variation under cultivation. Based on the nature of the panicle, shape, size of the fruit and other

general growth characters cardamom is broadly grouped into three main 'cultivated types' – Malabar, Mysore and Vazhukka. The naming of commercial types based on the places of production has led to confusion regarding the identity of the varieties. All the varieties and races are inter fertile and the observed variations are probably due to natural crossing. Since environment plays an important role in the phenotype of an organism, the genetic variations cannot be measured by the phenotypic evaluation alone. DNA markers provide much clear insight into genetic variation at molecular level (Rafalski *et al*., 1996). The present study aims to use molecular data to augment the current understanding of diversity and inter relationships between the cultivated cardamom and some of the related genera.

MATERIALS AND METHODS

Plant material

Ninety six important collections of *Elettaria cardamomum* var. *minor* including varieties, promising lines and other cultivated types representing most of the diversity available in cardamom germplasm conserved at different cardamom research stations were included in the present study (Table 1). Eleven species representing 5 major genera *viz., Amomum subulatum, A. aromaticum, A. ghaticum, A. microstephanum, A. involucratum, Alpinia galanga, A. purpurea, A. mutica, Aframomum melegueta, Hedychium coronarium* and *Elettaria cardamomum* were used to study the interrelationship among the closely related taxa of cardamom (Table 1).

DNA isolation and ISSR, RAPD & PCR- RFLP assay

Approximately 2 g of cardamom leaf tissue was collected from healthy plants and frozen in liquid nitrogen. Genomic DNA was isolated according to the protocol of Murray and Thompson (1980) with slight modification and dissolved in 250 µl of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA pH 8.0). The concentration of DNA samples were determined by running 1µl of samples with standard (25µl) undigested DNA (Sambrook *et al.* 1989) on a 0.8% Agarose gel in Tris Borate buffer. Hundred random primers *viz*., OPA – OPF series obtained from OPERON Technologies, Alameda, California, US were screened for amplification and polymorphism, of which 30 RAPD primers were used for profiling of related genera of cardamom and 50 RAPD primers were used for released varieties and important cultivars. RAPD reactions were performed according to the protocol of Williams *et al*. (l990) in a 25 µl reaction mix containing 0.15 mM dNTPs (Pharmacia Biotech), 2 mM $MgCl_2$, 30 ng genomic DNA, 10 picomoles of primers, and of 1U Taq DNA polymerase (Genei. Bangalore). The amplification were carried out in a Perkin Elmer 9700 thermal cycler with initial denaturation at 94 °C for 3 minute and followed by 30 cycles of 94 °C for 1min., 40 °C for 1minute 72 °C for 1minute and the final extension of 72 °C for 10 min. ISSR reactions were carried out with dinucleotide repeat primers (Synthesized from Genei. Bangalore) with two 3^{l} anchored primers (Wolfe and Liston 1998). Thirty picomoles of primers were used for single 25µl reaction. Primer-annealing temperature was raised to 50 °C, other PCR conditions were same as for RAPD assay. 12 primers viz., $(CT)_7$ TG, $(CT)_7$ AG, $(CT)_7$GC, $(CA)_7$ AC, $(CA)_7$ GT, $(CA)_7$ AG, $(CA)_7$ GG, $(GA)_6$ GG, $(GA)_6$ CC, $(GT)_6$ CC , $(GT)_6$ GG and $(CAC)_3$ GC were used for the profiling of related genera of *E. cardamomum*, 6 primers viz., $(CT)_7$ TG, $(CT)_7$ AG, $(CT)_7$ GC, $(CA)_7$ AC, $(CA)_7$ AG, $(CA)_7$ GG were used for the profiling cardamom collections. In view of the limited polymorphism observed in RAPD profiles of cardamom-cultivated types a modified RFLP PCR was standardized. RAPD reaction was carried out in 40 µl volume as per the standard conditions for cardamom. 15 µl of the amplified product was loaded in 2% agarose gel in TBE Buffer and the rest was used for restriction digestion. The product was digested with Eco R1 (New England Biolab) in a reaction containing 5 µl of Eco R1 Buffer, 0.15µl of enzyme (20,000 U), 19.85 µl of sterile double distilled water, and 25 µl of amplified product in a PCR tube and incubated at 37 °C for 3 hours. The digested samples were resolved on a 2% agarose gel in 1XTAE buffer stained with Ethidium bromide (0.5 mg/ml).

Construction of phylogenetic tree

The presence or absence of bands from ISSR, RAPD and PCR- RFLP profiles were scored as '1' (Presence) and '0' (absence). The data was combined into a single matrix. The cluster analysis was performed according to the Unweighted Pair Group Method with Arithmetic Average (UPGMA) method. Dendrogram was created for both cultivated types and related genera using NTSYSpc2.0 software for studying genetic distances and phylogrames.

RESULTS AND DISCUSSION

In the present study molecular profiling of 96 collections of cardamom germplasm representing the range of genetic variability and 11 species representing 5 major genera viz., *Amomum, Aframomum, Alpinia, Hedychium* and *Elettaria* were studied using RAPD, PCR - RFLP and ISSR polymorphism.

Molecular characterization of different collections of cardamom (*Elettaria cardamom* var. minor)

When the collections of cardamom germplasm, which include among others, released varieties; promising lines

Table 1: Germplasm collections of cardamom included in the present study

No	Genotype	Plant type	Characteristics
1.	Mudigere-1[3]	Malabar	Compact plant, suitable for high density planting. Short panicle, oval bold pale green capsule. Suitable for traditional cardamom growing malanad areas of Karnataka.
2.	Mudigere-2[3]	Malabar	An early maturing type. Round/oval and bold capsules. Suitable for cardamom growing tracts of Karnataka.
3.	PV-1[5]	Malabar	An early maturing variety, long panicles, oblong bold and parrot green capsule. Suitable for all cardamom tracts of Kerala.
4.	PV-2[5]	Vazhukka	Ovoid and parrot green capsules, thin fruit wall, 23% dry recovery tolerant to biotic stress like thrips, clump rot and capsule rot. Suited for cardamom Hill Reserve of Idukki district.
5.	ICRI-1[4]	Malabar	An early Maturing profusely flowering variety, medium sized panicles, globose, extra bold dark green capsule.
6.	ICRI-2[4]	Mysore	Suitable for higher altitudes, medium long panicles oblong bold and parrot green capsules. Tolerant to azhukal disease.
7.	CCS1[1]	Malabar	An early maturing variety with bold capsules. Suitable for high density planting.
8.	RR1[1]	Malabar	Dark green capsule, shoot/ panicle/ capsule borer (*Conogethes puncitferalis*) tolerant Rhizome rot (*Pythium vexans* and *Rhizoctonia solani*) resistant OP progeny of CCS-1. High yielder, suitable for planting in valleys of Karnataka and Waynad.
9.	RR2[1]	Malabar	Rhizome rot escape, high biomass type, short triangular capsule.
10.	NKE9[1]	Malabar	Resistant to mosaic, pubescent.
11.	NKE12[1]	Malabar	High yielding, bold capsule. Resistant to mosaic, pubescent. Field Tolerant to thrips (*Sciothrips cardamom*). Shoot/panicle /capsule borer (*Conogethes puncitferalis*).
12.	NKE 19[1]	Malabar	Resistant to mosaic, pubescent.
13.	Sampajie[1]	Malabar	High yielding, leaf pubescent, round capsule.
14.	Green Gold[1]	Mysore	Land race. Dark green bold capsules.
15.	MCC 260[4]	Vazhukka	High yielding type, planters selection.
16.	PS27[5]	Malabar	High yielding round capsule tolerant to thrips.
17.	S1[5]	Malabar	Bolder than PV1, oblong fruit, consistent yield.
18.	SKP51[2]	Malabar	Collection from N.Kannara.
19.	SKP100[2]	Malabar	Collection from N.Kannara.
20.	SKP165[2]	Malabar	Collection from N.Kannara, oblong bold capsule, long panicle.
21.	SKP170[2]	Malabar	Collection from N.Kannara, bold capsule, high yielder.
22.	Wonder cardamom[1]	Malabar	Suitable for low elevation.
23.	Pallakkodi[1]	Malabar	High yielder.
24.	NKE 4[1]	Malabar	Resistant to mosaic, pubescent.
25.	NKE 8[1]	Malabar	Resistant to mosaic, pubescent.
26.	NKE 11[1]	Malabar	Resistant to mosaic, pubescent.
27.	NKE 16[1]	Malabar	Resistant to mosaic, pubescent.
28.	NKE 26[1]	Malabar	Resistant to mosaic, pubescent.
29.	NKE 28[1]	Malabar	Resistant to mosaic, pubescent.
30.	NKE 31[1]	Malabar	Resistant to mosaic, pubescent, elongated capsule.
31.	NKE 32[1]	Malabar	Resistant to mosaic, pubescent.
32.	NKE 34[1]	Malabar	Resistant to mosaic, pubescent.
33.	NKE 71[1]	Malabar	Resistant to mosaic, pubescent.
34.	NKE 72[1]	Malabar	Resistant to mosaic, pubescent.
35.	NKE 78[1]	Malabar	Resistant to mosaic, pubescent.
36.	Compound panicle 3[1]	Malabar	Collection from Kodagu.
37.	Compound panicle 4[1]	Malabar	Collection from Kodagu.
38.	Compoundpanicle 10[1]	Malabar	Collection from Kodagu.
39.	Compoundpanicle11[1]	Malabar	Collection from Kodagu.
40.	Compoundpanicle12[1]	Malabar	Collection from Kodagu.
41.	Multibranch 3[1]	Malabar	Branched panicles, collection from Kodagu.
42.	Multibranch 25[1]	Malabar	Branched panicles, collection from Kodagu.
43.	Multibranch 26[1]	Malabar	Branched panicles, collection from Kodagu.
44.	Multibranch 27[1]	Malabar	Branched panicles, collection from Kodagu.
45.	Multibranch 29[1]	Malabar	Branched panicles, collection from Kodagu.
46.	Multibranch 37[1]	Malabar	Branched panicles, collection from Kodagu.

Contd.

No	Genotype	Plant type	Characteristics
47.	Multibranch 45[1]	Malabar	Branched panicles, collection from Kodagu.
48.	Multibranch 60[1]	Malabar	Branched panicles, collection from Kodagu.
49.	MCC 12[4]	Mysore	Deep green capsule, occasional top bearing panicle.
50.	MCC 21[4]	Vazhukka	Long bold capsule, Highly adapted to Idukki tract.
51.	MCC 40[4]	Malabar	Early bearing, long capsule.
52.	MCC 61[4]	Mysore	Long bold, Parrot green capsule, relative tolerant to capsule rot.
53.	MCC73[4]	Malabar	High yielder, angular bold capsule.
54.	MCC 85[4]	Vazhukka	Angular bold capsule.
55.	MCC346[4]	Vazhukka	Well adapted to all cardamom growing areas, angular bold capsules.
56.	Malabar 20	Malabar	High biomass type, short green elongated capsule, drought tolerant type.
57.	Malabar 21	Malabar	Boldest and elongated capsule in Malabar, leaf pubescent.
58.	Malabar 22	Malabar	High biomass type, ovoid capsule.
59.	Malabar 25	Malabar	High yielding collection.
60.	Malabar 27	Malabar	High yielding collection.
61.	APG 8[1]	Malabar	Collection from Kodagu.
62.	APG 10[1]	Malabar	Collection from Mudigere.
63.	APG 21[1]	Malabar	Collection from Mudigere.
64.	APG 27[1]	Malabar	Collection from Mudigere.
65.	APG 28[1]	Malabar	Collection from Mudigere.
66.	APG 36[1]	Mysore	Collection from Pampadumpara.
67.	APG 41[1]	Mysore	Collection from Pampadumpara.
68.	APG 43[1]	Malabar	Collection from Mudigere.
69.	APG 52[1]	Malabar	Collection from Pampadumpara.
70.	APG 72[1]	Malabar	Collection from Koppa.
71.	APG 76[1]	Malabar	Collection from Yeslore.
72.	APG 100[1]	Malabar	Collection from Gowdahalli.
73.	APG 121[1]	Malabar	Collection from Valayar.
74.	APG 168[1]	Malabar	Collection from Burliar.
75.	APG 242[1]	Malabar	Collection from Kodagu.
76.	APG 82[1]	Vazhukka	Collection from Kodagu.
77.	APG 62[1]	Mysore	Collection from Mudigere.
78.	APG 50[1]	Malabar	Collection from Pampadumpara.
79.	APG 48[1]	Mysore	Collection from Nenmara.
80.	APG 86[1]	Mysore	Collection from Mudigere.
81.	APG 316[1]	Vazhukka	Collection from Kodagu.
82.	APG 282[1]	Vazhukka	Collection from Kodagu.
83.	APG 256[1]	Vazhukka	Collection from Kodagu.
84.	Vazhukka 9[1]	Vazhukka	High yielder.
85.	Hybrid 3[1]	Vazhukka	High yielder hybrid.
86.	Hybrid 5[1]	Malabar	High yielder, long panicles.
87.	MHC 10[4]	Mysore	Hybrid progeny.
88.	MHC 13[4]	Mysore	Hybrid progeny.
89.	MHC 18[4]	Vazhukka	Hybrid progeny.
90.	MHC 26[4]	Malabar	Hybrid progeny.
91.	MHC 27[4]	Mysore	Hybrid progeny.
92.	Minipink[5]	Malabar	Light pink coloured stem, very light pink panicles, showing slight resistance to thrips.
93.	Pink base[5]	Malabar	Pink panicle and clump.
94.	Narrow Leaf[1]	Malabar	Narrow elongated leaf.
95.	Multibranch Sterile[1]	Mysore	Branching type, Nelliampathy collection.
96.	Myladumpara Sterile[4]	Malabar	Sterile type.
97.	*Amomum subulatum*[1]	Large cardamom	Commercially grown in Nepal and Sikkim.
98.	*Amomum aromaticum*[1]	Bengal cardamom	Seeds are used as spice, in place of *A.subulatum.* Contains large quantities of cineole. Grown in West Bengal.
99.	*Amomum ghaticum*[1]	-	Rare and endangered species occurring in Indo Malayan region.
100.	*Amomum microstephanum*[1]	-	Leaves silky, tomaritose breath, leafy stem 4-5 ft long, and petiole 1-7 inches long, corolla lobes linear-oblong, ovary glabrous.

Contd.

No	Genotype	Plant type	Characteristics
101.	*Amomum involucratum*[1,]	-	Occurs in Sri Lanka and Anamalai hills in India, flowers numerous, lip pale ochraceous yellow.
102.	*Alpinia galanga*[1]	Greater galangal	Native to Indonesia, but found in many places of India, Sub Himalayan region of Bihar, West Bengal and Assam. Used as a reputed Drug in the indigenous system of medicine.
103.	*Alpinia purpurea*[1]	Red ginger	Moluccas to New Caledonia. Herb with leafy stem ending in a showy inflorescence with a brush like flower spike with numerous large boat shaped red bracts each with a white flower. Apomictic plant.
104.	*Alpinia mutica*[1]	Orchid ginger	Distribution -India and Malaysia.
105.	*Aframomum melegueta*[1]	Grains of paradise	Seeds contain 1-2% essential oil with 35.1% cineole as major component
106.	*Hedychium coronarium*[1]	Ginger Lilly,Garland flower	Native to India and East Indies. An essential oil obtained from rhizome is active against gram-positive bacteria and fungi; Powered rhizome are used in medicine. Decoction is considered as antichematic and tonic; Aerial stem are used for manufacturing of paper.

1.Indian Institute of Spices Research, Cardamom Research Station Appangala, Kodagu, Karnataka; *2.* Indian Cardamom Research Institute Sakleshpur, Karnataka; 3.University of Agricultural Sciences, Mudigere, Karnataka; 4.Indian Cardamom Research Institute, Myladumpara, Idukki district, Kerala; 5. Kerala Agricultural University, Center Pampadumpara, Idukki District, Kerala.

and local cultivars of cardamom were characterized using 50 RAPD primers, 6 ISSR primers and 2 PCR - RFLP primers, produced 220 polymorphic bands (Fig. 1). Phylogrames were created for studying genetic distance for all the genotypes (Fig. 2) and separately for 24 released varieties and new varieties in the process of development for better clarity (Fig. 3).

The phylogram generated (Fig 2) has shown that all the genotypes are well separated except NKE (Natural Katte Escape) NKE 8, NKE 16, NKE 4 and NKE 26 indicating that except for these 4 NKE lines all the other accessions are different and are not duplicates.

There are five major clusters formed in the phylogram. In

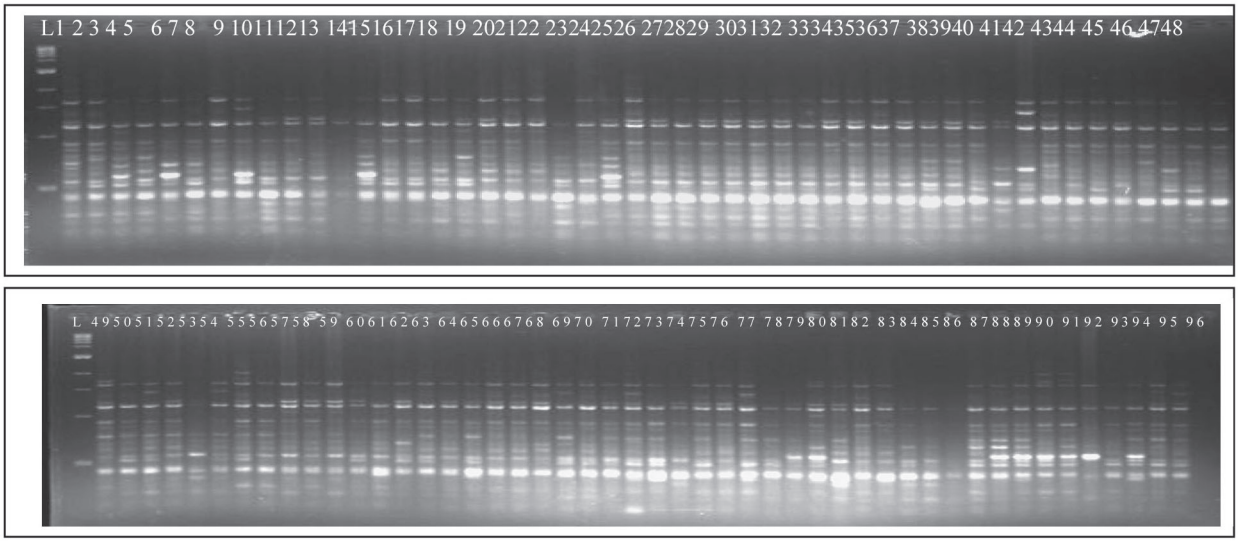

Fig.1: RAPD polymorphism of cultivated small cardamom, as collection generated by primer OPE 8

1.M1 2.M2 3.PV1 4.PV2 5.ICRI1 6.ICRI2 7.CCS1 8.RR1 9.RR2 10.NKE9 11.NKE12 12.NKE19 13.Sampajie Clone 14.Green Gold 15.MCC260 16.PS27 17.S1 18SKP51 .19.SKP100 20.SKP165 21.SKP170 22.Wonder cardamom 23.Pallakodi 24.NKE4 25. NKE8 26. NKE11 27. NKE16 28. NKE26 29. NKE28 30. NKE31 31. NKE32 32. NKE34 33. NKE71 34. NKE72 35. NKE78 36. CP3 37. CP4 38. CP10 39. CP11 40. CP12 41.MB3 42. MB25 43. MB 26, 44. MB 27, 45. MB 29, 46. MB 37, 47. MB 45, 48. MB 60, 49. MCC 12, 50.MCC 21, 51.MCC 40, 52.MCC 61, 53. MCC 61, 54. MCC 85, 55. MCC 346, 56. Malab20, 57.Malab21, 58.Malab22, 59.Malab25, 60.Malab27, 61.APG8, 62.APG10, 63. APG 21, APG27, 64. APG27, 65. APG 28, 66.APG36, 67.APG41. 68.APG 43, 69.APG52, 70.APG 72, 71.APG 76, 72.APG 100, 73. APG121, 74.APG 168, 75.APG 242, 76.APG 82, 77. APG 62, 78.APG50, 79.APG48, 80.APG 80, 81.APG 316, 82.APG 282, 83.APG 256, 84.Vazhukka9, 85. Hyb 3, 86. Hyb5, 87. MHC 10, 88.MHC13, 89. MHC18, 90.MHC 26, 91.MHC 27, 92. Minipink, 93. Pink base, 94.Naroow leaf, 95.Multibranch sterile, 96. Mylad. Sterile. L : 1kb Ladder

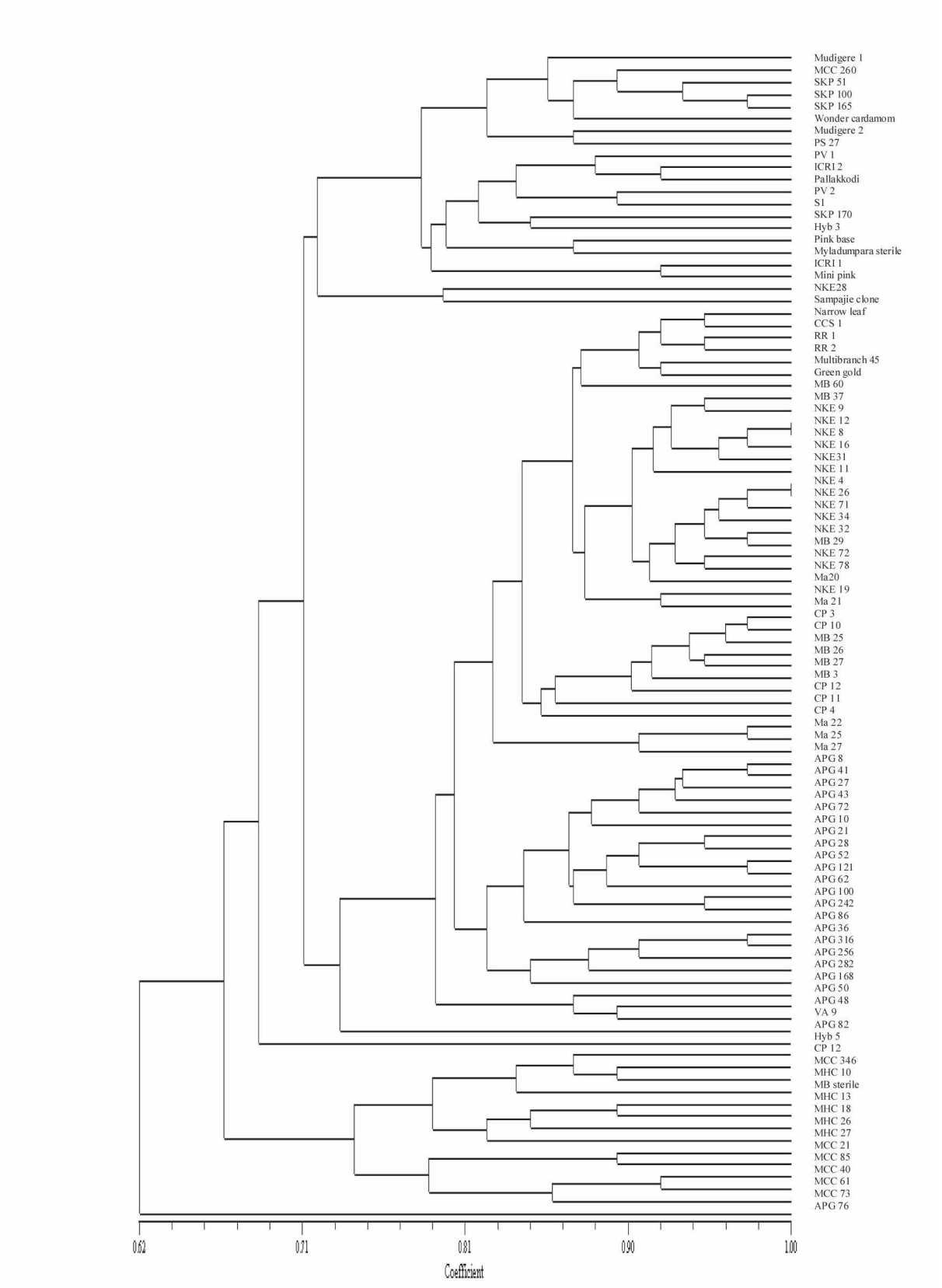

Fig. 2: Dendrogram of collections of *E. cardamomum* Maton. produced using UPGMA clustering of pair wise similarity distances using RAPD and RAPD-PCR polymorphism. Details of the genotypes are given in Table 1

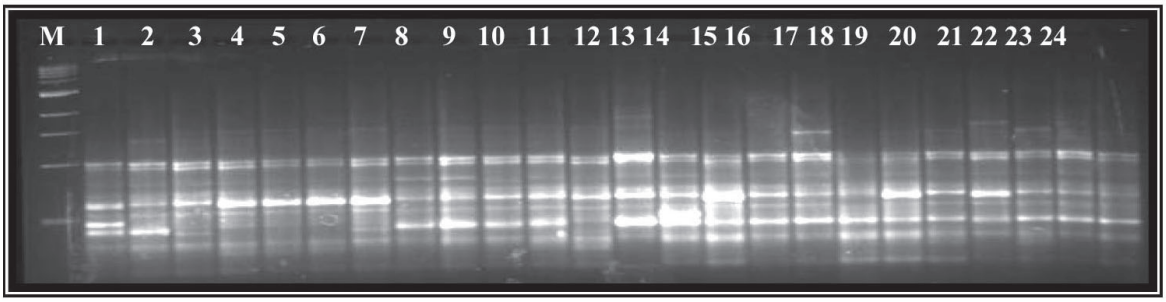

Fig. 3: ISSR polymorphism in released varieties and promising lines of *E. cardamomum,* generated by primer IS 6
Lane M. 1 kb ladder, 1.ICRI 1, 2... ICRI 2, 3.CCS 1, 4...RR 1, 5.NKE 9, 6.NKE 12, 7.NKE 19, 8.PV 1, 9. PV 2, 10.M 1, 11.M 2, 12.Sampajie, 13. MCC 12, 14. MCC 21, 15.MCC 40, 16. MCC 85, 17. MCC 346, 18. S 1, 19.PS 27, 20. SKP 165, 21. SKP 170, 22.HY3, 23. MHC 26, 24.MHC 27; **Primer IS 6 (5[1]-CACACACACACACAAG 3')**

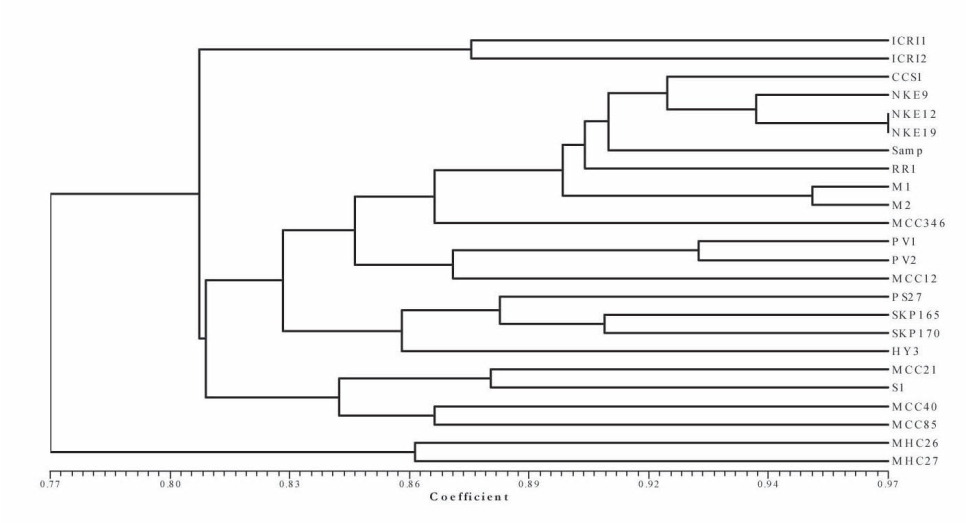

Fig. 4: Dendrogram of diversity showing similarity among the released varieties and promising lines of cardamom genotypes studied using ISSR polymorphism

general, collections from same center clustered together. This may indicate that location and population specific collections are maintained in each center *i.e.* most of the collections maintained in each center are segregants from one or two major populations.

Most of the collections from Indian Institute of Spices Research, Cardamom Research Center, Appangala, Karnataka formed the main central cluster with two major sub-groups. One comprising mainly of NKE lines (Venugopal,1999) and the other containing APG lines. The clustering of NKE and APG lines indicate their possible origin as segregating progeny from populations of narrow genetic diversity and collected possibly from same genetic stock.

The compound panicles and multi branch types are grouped into a different groups individuals indicating that this character has originated from more than one genotype from both Karnataka as well as Kerala collections. But the sterile multi branch line is uniquely placed. These collections with multiple branching and compound panicles have been reported to have higher yield potential (Miniraj *et al,* 2000).

PV1, PV2, PS 27, S1, minipink, pinkbase which was collected from KAU center Pampadumpara were grouped with Mudigere 1, Mudigere 2, ICRI 1, ICRI 2, SKP 50, SKP 100, SKP 165 and 170 and this cluster showed high amount of divergence from the major clusters.

Green gold (*Njallani*), a farmer's collection from Idukki district of Kerala, is distinctly different from other released varieties of cardamom. MCC 260, a planter's selection from Indian Cardamom Research Institute (ICRI) Myladumpara, 'Pallakodi' and 'Wonder cardamom', which are two other popular cultivated types, also showed distinct differences.

Separate analysis of all the released varieties and promising lines from different centers (Fig 2 and 4) also showed similar patterns. The varieties developed from each center tend to

cluster together. All these indicate clear divergence in Kerala and Karnataka collections, the two main regions of cardamom diversity. The comparatively less divergence within a population is because of the open pollinated seed origin (siblings) of the individual collections. It may be noted that most of the cardamom plantations are planted with seed derived plants with reasonable amount of uniformity and still seed progeny is the preferred method of propagation though clonal propagation is catching up.

The collections and hybrids from ICRI Myladumpara formed one cluster and they are well separated from rest of the genotypes and most of them are of hybrid origin. This indicates controlled breeding rather than selection from open pollinated progeny is to be preferred in cardamom to bring more variability in germplasm. Prasath *et al* (2004) reported high variation among cultivated small cardamom accessions but this was not reflected in molecular characters.

Interrelationship among the related genera of cardamom

When eleven species representing five major genera viz., *Amomum subulatum, A. aromaticum, A. ghaticum, A. microstephanum, A. involucratum, Alpinia galanga, A. purpurea, A. mutica, Aframomum melegueta, Hedychium coronarium* and *Elettaria cardamomum* were profiled for polymorphism using 30 RAPD and 12 ISSR primers, high amount of RAPD and ISSR polymorphism was noticed (Fig 5).

In the present study, the phylogram generated (Fig 6) showed that all the genotypes are separated at similarity

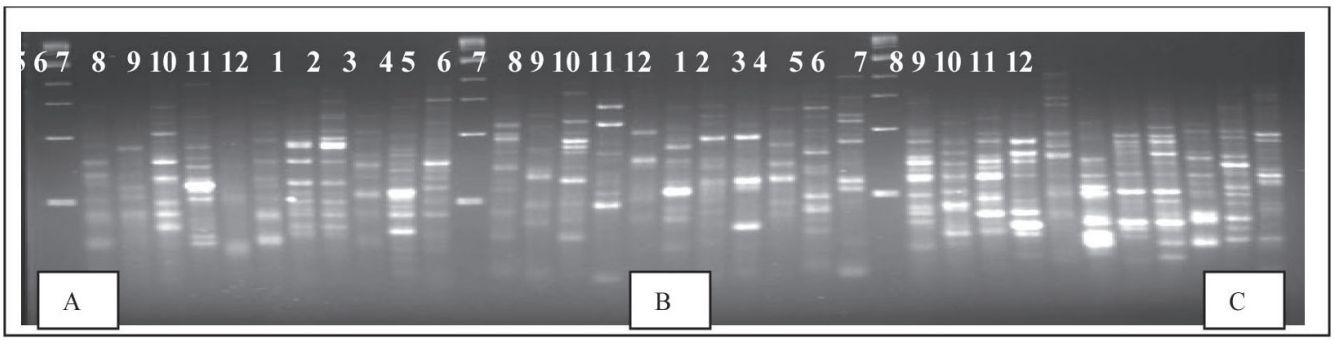

Fig. 5: ISSR polymorphism in wild and related genera of *E.cardamomom* as expressed by primers
Lane*1*. 1 kb ladder, 2.*Amomum subulatum*, 3. *A. ghaticum*, 4. *A. microstephanum*, *5. A. melegueta*, 6. *A. involucratum*, 7. *Amomum* aromaticum, 8. *Alpinia purpurea*, 9. *A. galangal*, 10. *A. multicar* 11.*Hedychium coronarium*, 12. *Elettaria cardamomum* (APG 6)

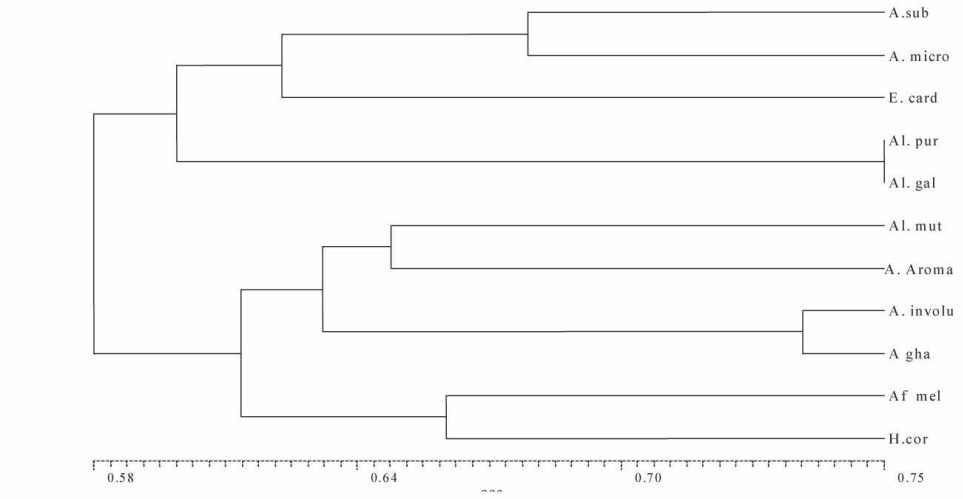

Fig. 6: Dendrogram of diversity showing similarity among the related genera of cardamom genotypes based on ISSR polymorphism.

coefficient between 58 and 75%. Two major clusters were formed; *E.cardamomum* formed a first cluster with *Amomum subulatum, A.microstephanum* (62.8% similarity) and *Alpinia purpurea, A. galanga* (60% similarity). This group linked to the second major cluster with 58% similarity. The second cluster comprised of *Alpinia mutica, Amomum aromaticum, A. involucratum, Alpinia galanga, Aframomum melegeuta* and *H.coronarium*. The recent classification proposed by Kress *et al.* (2002), based on the DNA sequences of Internally Transcribed Spacer region (ITS), grouped *Alpinia, Aframomum, Amomum* and *Elettariopsis* (which is near to *Elettaria*) in a single clade 'Alpineae', whereas *Hedychium, Zingiber* and *Curcuma* formed another clade 'Zingibereae'.

Elettaria cardamomum is clustered with *Amomum subulatum* and *A. microstephanum* indicating that *Amomum* is closery to cultivated cardamom among the genera studied. The molecular profiles indicated clear clustering together of most of the species of each genus except *Alpinia mutica* and *Amomum aromaticum* that formed separate cluster. This is in agreement with Fischer (1956) who in his classification, of the family Zingiberaceae, has indicated similarity of *Elettaria* to *Amomum* based on the absence of lateral staminodes (which are small and narrow or absent) and to *Alpinia* based on the inflorescence type.

The Zingiberaceae is distributed pantropically and is still poorly known taxonomically with many species. The currently accepted classification of the Zingiberaceae includes four tribes Hedychiaea twenty two genera, Alpinieae twenty five genera, Zingibereae one genus, Globbeae four genera, and is based on both vegetative and floral characteristics. Nearly one-quarter of the genera in the Zingiberaceae are monospecific, but no criteria for the recognition of these species at the generic level have been established. Studies using molecular sequence data (ITS- nrDNA and *trn*l–f -cpDNA, *matK*) have been utilized to generate hypotheses on the phylogenetic relationships among the genera of the Zingiberaceae to evaluate past classifications of the family, to identify morphological features that characterize the various clades and can be detected by molecular analyses, to evaluate the status of monotypic genera where ever possible, and to construct a new phylogenetic classification of the family (Kress *et al*., 2002; Ngamriabsakul *et al,* 2003; Nirmal Babu *et al.*, 2005, 2011).

The present study indicates that Elettaria is closer to *Ammomum* and *Alpini*a which is in agreement with the modern classification of Kress *et al.* (2002) based on ITS sequences.

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Genome specific primers: A tool for genetic profiling of potato species

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ABSTRACT

Genome sequencing of the *Solanum phureja* (a diploid species of potato) has provided new insights to understand the complete genome functioning of the *Solanum* species. The present study was conducted to understand the genetic relatedness among different potato species. A set of twenty genotypes, including wild and cultivated, possessing agonomically important traits were analyzed for genetic diversity using the genome specific markers. Twenty randomly selected BAC end sequences were used for primer designing and screening. Expectedly, the commercial potato cultivars along with their progenitors used in the present study were clustered. The wild species viz., *S. albicans, S. sleumeri* and *S. demissum* clustered separately in the dendrogram indicating that these are genetically unrelated with the rest of the species. Result of the principal component analysis (PCA) was consistent with the clustering pattern of the dendrogram. The generation of the genetic profiling of the potato species using the genome specific markers will help in documentation of ownership and protection of intellectual property rights along with the presently used RAPD, AFLP and SSR profiles.

Key words: *Solanum sp*., genome, primers, diploid, tetraploid, PCA

INTRODUCTION

The cultivated potato, *Solanum tuberosum* L. (2n=2x=24) is a member of an economically important family Solanaceae with more than 3000 species. Cultivated potato has a narrow genetic base owing to the fact, that present day cultivars evolved from a narrow genepool introduced from few germplasm sources of South American centre of origin to the new world and Asia during the western colonial rule. The potato genome consists of 12 chromosomes and has a (haploid) length of approximately 844 million base pairs, making it a medium-sized plant genome. The genome of potato has been sequenced by international consortium of 26 institutes from 13 countries as part of a larger initiative called the 'Potato Genome Sequencing Consortium' (Xu *et al.,* 2011). Along with whole genome sequence other genomic resources for potato have been developed including Expressed Sequence Tag (ESTs) (Rensink *et al.,* 2005), Bacterial Artificial Chromosome (BAC) clone, phasmid libraries (Chen *et al.,* 2004), microarray platforms (Kloosterman *et al.,* 2005) and a dense genetic map (Van *et al.,* 2006). These resources have been utilized in studies on potato physiology, development, responses to abiotic and biotic stress, polyploidy, comparative genomics as well as enhancement of genetic maps (Kloosterman *et al.,* 2005; Rensink *et al.,* 2005). Many studies have shown relationship among different species using comparative genome mapping (Paterson *et al.,* 2000), reconstruction of ancestral genomes (Blanchette *et al.,* 2004), phylogenetic studies (Rokas *et al.,* 2003), deciphering patterns of natural selection on coding regions (Bustamante *et al.,* 2005), and predictions of common gene function across species (Doganlar *et al.,* 2002) in potato. While the cultivated species have been bred for these diverse agronomic traits, genome sequence analysis has indicated that these species share, to a large extent, not only genes (Xu *et al.,* 2011) but also gene order (synteny) between their genomes (De Jong *et al.,* 2004; Xu *et al.,* 2011). While major classes of repetitive sequences are conserved among some Solanaceae species (Ganal *et al.,* 1988; Schweizer *et al.,* 1993), lineage-specific repetitive sequences have been reported, suggesting that divergence of this fraction of the genome has occurred through evolution. The availability of the whole genome sequence data would provide opportunities for exploring DNA-level diversity among the members of a crop species and its relationship to phenotypic diversity. Now the potato genome is sequenced, the next important question for the researchers would be utilization of this genome information to study various agronomically important genes present in different wild species and to get them into the cultivated tetrapliod potato.

Would the sequenced diploid potato (*S. phureja*) data help in studying the other species? If yes, to what extent? How far different potato species are inter-related? Which species of potato are near relative and which are distant relative? To know the answers for these questions a genetic profiling study was conducted using genome specific markers and 20 different potato genotypes including wild, semi-cultivated and cultivated possessing agronomically important traits.

MATERIALS AND METHODS

Plant material

Twenty potato (*Solanum spp.*) genotypes including wild, semi-cultivated and cultivated species were evaluated. Tubers of 20 potato genotypes (Table 1) including few commercial cultivars and an advanced hybrid which possess agronomically important traits were grown under the poly-house conditions in earthen pots during summer, 2010.

DNA extraction and quantification

Total Genomic DNA from the tender leaves of each potato plant was isolated using Sigma kit (GeneElute™ Plant Genomic DNA Miniprep kit) and genomic DNA was quantified using the spectrophototmeter (UV-1700 PharmaSpec, Shimadzu) and quality was checked both by A260/A280 ratio and by gel-electrophoresis. Working stock of 300 ng/ul was made for using in PCR reactions.

Primer designing

Randomly 20 BACs were selected from the Potato BAC end sequence library, 10 each from DM (Double Monoploid, *S. Phureja*, DM1-3 516 R44) and RH (*S. tuberosum*, RH89-039-16) BAC end libraries from the PGSC site (www.secure.potatogenomics.com). The genomic sequence from each BAC end sequence was used to design both forward and reverse primer set using Primer3 software (http://frodo.wi.mit.edu/primer3/). The primers were designed to get amplicon size ranging from 350 bp to 700 bp, with 24 to 30 nucleotide length, having high annealing temperature (Tm) and > 50% GC content in the amplicons to ensure high specificity while amplification. These primer sequences were synthesized from M/S Lab India (IDT) (Table 2).

PCR conditions

All PCR reactions were carried out with total reaction volume of 20 µl containing 1 µl (300 ng) of genomic DNA along with 200 µM dNTPs, 0.2 µM both forward and reverse primer, 2 units Taq polymerase (AmpliTaq, Applied Biosystems) and 1.5 mM Mg^{2+} was used in the PCR reaction of 35 cycles in thermocycler (Gene Amp PCR system 9700

Table 1: Selected genotypes (*Solanum* species) and their importance

S. No.	Species (Clone No.)	Importance	Selected References
1.	*S. alandiae* (SS 1764-01)	Low cold induced sweetening	Bhardwaj *et al.,* 2011
2.	*S. albicans* (SS 1763-18)	Low cold induced sweetening	Bhardwaj *et al.,* 2011
3.	*S. andigena* (JEX/A-85)	Black dot resistance Cyst nematode resistance Late blight	Nadav *et al.,* 2010
	S. andigena (JEX/A-1038)	resistancePVX resistance	Gabriel *et al.,* 2007
4.	*S. balbocastinum* (SS 1721)	Late blight resistance	Song *et al.,* 2003
5.	*S. brevicaule* (SS 1794-07)	Low cold induced sweetening. Late blight resistance	Bhardwaj *et al.,* 2011
6.	*S. cardiophyllum* (SS 1825-10)	Late blight resistance	Bradshaw and Ramsay, 2005
7.	*S. chacoense* (SS 660-16)	Leptin rich, Colarado beetle resistant, PVX and PVY resistant	Susannah *et al.,* 2009; Bradshaw and Ramsay, 2005
8.	*S. demissum* (SS 1850-4)	Cytoplasmic sterility. Late blight resistance	Bradshaw and Ramsay, 2005
9.	*S. jamesi* (SS 1652-09)	Low cold induced sweetening	Luthra *et al.,* 2009
10.	*S. mona* (SS1659-02)	Not studied	-
11.	*S. sleumeri* (SS 1301-04)	Not studied	-
12.	*S. sparsipilum* (SS 1724-2)	Late blight resistance. Root knot nematode resistance Cyst nematode resistance	Finbarr *et al.,* 2010; Kouassi *et al.,* 2005 Katella *et al.,* 2007
13.	*S. spegazzinii* (SS 1725-84)	Cyst nematode resistance. Late blight resistance	Bradshaw and Ramsay, 2005; Paal *et al.,* 2004
14.	*S. tuberosum* ssp. *tuberosum*		
14.1	Atlantic	First exotic high yielding variety	CPRI bulletin
14.2	Kufri Chipsona-III	High yielding, Good processing quality, Late blight tolerant	CPRI bulletin
14.3	Kufri Pukhraj	High yielding, Early blight tolerant and moderately tolerant to late blight	CPRI bulletin
14.4	Phulwa	Parentage of K. Deva and K. Sheetman, K. Safed, K. Chandramukhi	CPRI bulletin
14.5	Kufri Badshah	High yielding, Early and Late blight tolerant	CPRI bulletin
14.6	MP/97-1008	Used as parent in breeding programmme	CPRI bulletin

Table 2: List of genome primers used in the study

Sl No.	Primer	Sequence (5'—> 3')	Amplicon size (bp)
1.	RH 1	F: GCCTGAGCAATCTGCACAGGTC	
		R: CTGGCAAACGACGTCGGCCAAGT	515
2.	RH 2	F: TGATGGTGGTCCAAACCATGGAGA	
		R: TTGCTGGAAATAGGCCTGCT	358
3.	RH 3	F: GAGGAGTGCCTGCTGGAGCT	
		R: TGAGCCAATCCTTTTGGTGAGC	428
4.	RH 4	F: TGAGTTGACCTTCCTTGGTTGTCG	
		R: GAACTCAATGTTGATCGGGTTCTG	164
5.	RH 5	F: CTTTAGGCCTTGGAACCCTT	
		R: GTTTGCGAGCTATTGCATAGGAGC	216
6.	RH 6	F: GTGAGGGGTTTATACCTTGCCACC	
		R: GGGGCCATGACATTCTACCGGCTC	480
7.	RH 7	F: ATCGAGGGGTTCATCCGAATCTCC	
		R: ACACACCGAACCCTACTTGACCCT	448
8.	RH 8	F: TTGGGGAGCACAATTGCTTCCACC	
		R: GATCGGATTCCTTGGTTCGCAGA	256
9.	RH 9	F: TTTCGCAAGGATCGTCGTGGTA	
		R: AGCATGCTCGACACCCGAAC	478
10.	RH 10	F: AGGCTGTTGATGCTCACACT	
		R: GGAAGGCCAATATTGTAGCGGATG	512
11.	DM 1	F: TGAACTGCTCCAAGCAACTG	
		R: ATTCCAACGCACATGCCACA	356
12.	DM 2	F: ACTACAGGGATGAACCCAATCC	
		R: CTACGACACAATCAAACCCGA	367
13.	DM 3	F: ACGACTCGGTAACAGATCGCTT	
		R: TGGGGATTCTTGATTGGTGCTG	260
14.	DM 4	F: GAGGCTTACCTCAGCCACTG	
		R: GAAGTTACCCAAGGGCAGGT	597
15.	DM 5	F: TGGGGATCTTCACTAGTCTGCGA	
		R: GCTTATGCCAGGATTGCGAATGC	245
16.	DM 6	F: TCCTCAAGGCAGTATGGTGCTACG	
		R: ATGACTGGCTCACTAGGTTGCTCA	441
17.	DM 7	F: TTTCAGCGGCGGGCTTACCT	
		R: GATCCTTGCGCCGATTCTCG	519
18.	DM 8	F: TGTCCAACCCGTACGGCCAAAG	
		R: ACCGGTCACAACATCCAGAGAGCT	141
19.	DM 9	F: CACGCATCGATAGGGAACCCA	
		R: AGCTTCGACGAGCTGGGACGT	430
20.	DM 10	F: GCTTGCTCGAGAGAGAGAGGTCGT	
		R: CCGCATGAAGACTAGCCTATGGCT	163

from Applied Biosystems) by using the following temperature sequence: 94 °C for 1 min, 35.5 °C for 1 min and 72°C for 2 min. Cycles were preceded by denaturation for 5 min at 94 °C followed by a final extension at 72 °C for 10 min. The PCR product was run on 2.5% agarose gel with TAE as tank buffer and stained with ethidium bromide. Gel image was captured and stored in a computer using Fluor-S™ Multimager (Bio-Rad) (Fig. 1). Manual scoring was done for the presence or absence of the amplicons.

Data Analysis

DNA amplification with each primer was assayed twice. DNA fragment profiles were scored in a binary fashion with 0 indicating absence and 1 indicating presence of a band. A similarity matrix on the basis of band sharing was calculated from the binary data using Dice coefficient (Nei & Li, 1979). Similarities were graphically expressed using the group average agglomerative clustering to generate dendograms. The analysis was done using the software package NTSYS-pc (version 2.02) (Rohlf, 1998). Principal Component Analysis (PCA) from the binary data was performed to graphically summarize associations among the lines. Analysis was done through a batch file following the software package NTSYS-pc.

RESULTS AND DISCUSSION

The importance of divergence in the improvement of crop has been reported both in self and cross-pollinated crops

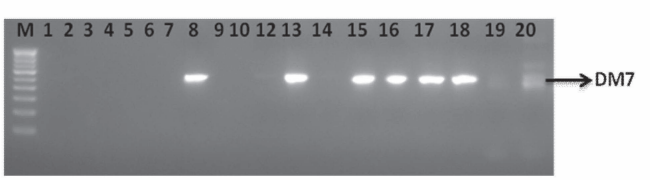

Fig. 1: Genome specific marker DM7 screening with 2.5% agarose (M-100bp ladder, 1-20 potato genotypes as mentioned in the table 1)

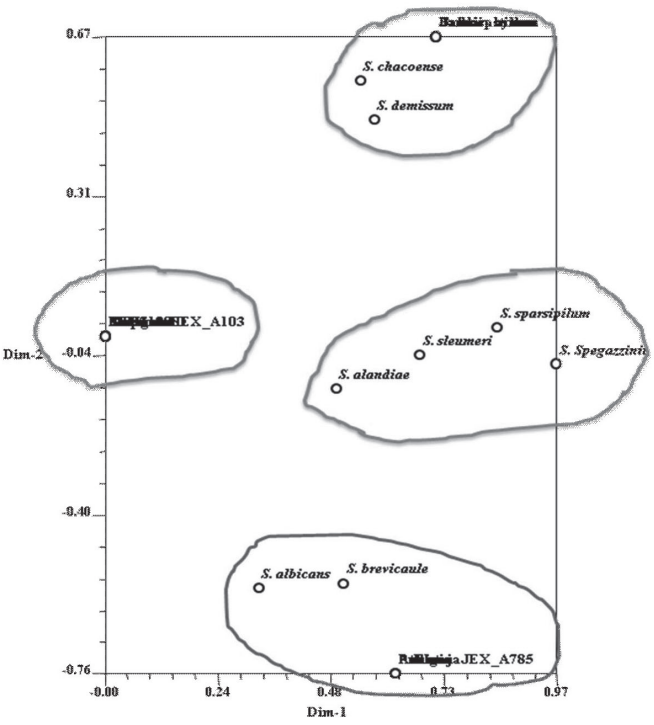

Fig. 2: Principal coordinate analysis (PCA) obtained by genome specific markers

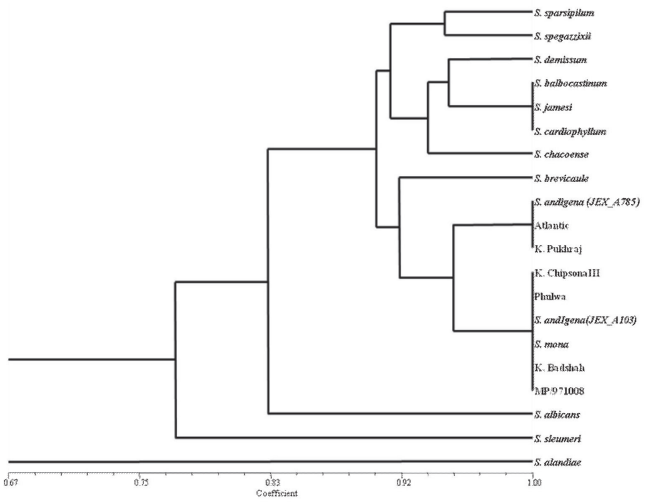

Fig. 3: Dendrogram depicting the phylogenetic relationship among potato genotypes

(Griffing and Lindsstromm, 1954; Gaur *et al.*, 1978). In the present study, 20 different potato genotypes including cultivated, semi-cultivated and wild species were compared using genome specific markers to study the genetic relatedness between the species. Coefficients of similarity were calculated according to Nei and Li (1979) and the matrix of similarity was analysed by the Unweighted Pair-Group Method (UPGMA). The associations among the species and cultivars were characterized by a multivariate technique, principal component analysis (PCA). The Principal Coordinate Analysis, where the first two principal components accounted for 76.1% of the variance, was able to separate the different groups. The PCA results (Fig. 2) indicated that all genotypes can be grouped into four major clusters. Besides, a dendrogram was constructed based on agglomerative clustering (Fig. 3) which also showed four major clusters confirming the PCA results. Clusters I, II and III contain one genotype each suggesting that these three wild species viz. *S. albicans*, *S. sleumeri* and *S. demissum* are un-related with other studied species. Cluster IV was the biggest cluster which further divided into four sub-groups consisting of both cultivars and wild species. In the pedigree of world potato cultivars *S. demissum* and *S. andigena* has been used to the maximum extent (Bradshaw *et al.,* 2006). Two commercial cultivars viz. Atlantic and Kufri Pukhraj closely clustered with *S. andigena* (JEX-A-785), a neo-tuberosum species which is one of the common parents in hybridization programme of these cultivars. Cultivars, Kufri Chipsona-III, Kufri Badshah, Phulwa and an advanced hybrid MP/97-1008 also closely clustered with *S. andigena* (JEX-A-103). In all these potato cultivars different *S. andigena* clones have been used as one of the progenitor in their breeding programmes and therefore the results suggest that these genome specific primers are able to distinguish among wild and cultivated species. Interestingly, *S. jamesi*, which is largely under-studied species, closely clustered with wild species *S. bulbocastanum*, *S. cardiophyllum* and *S. demissum*. This indicated that *S. demissum* is closely related to these three diploid, 1EBN species which are not easily crossable with cultivated *S. tuberosum.* It also largely indicates that all the agronomically important traits present in these three 1EBN species might have been taken care by *S. demissum* in the traditional potato breeding programmes. This study also gives an important clue that closely related wild species viz., *S. cardiophyllum*, *S. bulbocastanum*, *S. jamesi*, *S. sparsipilum* and *S. spegazzinii* share a common genetic pool of many agronomically important genes. It is also evident from the study that broad genetic diversity is present in unexploited wild species which can be further utilized for

crop improvement programmes to widen the genetic base of the commercial potatoes (Gopal and Oyama, 2005). The narrow genetic base among commercial cultivars in the study is expected as the markers are genome specific, besides, few markers and low sample size used in the present study. This is in contrast to the wide genetic base of Indian potato cultivars estimated using random amplified polymorphic DNA (RAPD) analysis (Chakrabarti *et al.*, 1999; Chakrabarti *et al*., 2001; Pattanayak *et al.*, 2002). This may be due to nonspecific amplification, lower annealing temperature, smaller primer size, involvement of entire genomic DNA in RAPD amplification. However, results obtained in the present study were in conformity with those obtained by Chimote *et al.* (2004), using simple sequence repeats (SSRs) for studying the diversity among the Indian potato cultivars. The genetic diversity revealed by genome specific markers may not present the actual genetic relationship among the various wild/cultivated *Solanum* species studied as similarity coefficient is based on presence or absence of a amplicon and the potato genotype having an allele in single, double, triple or quadruple doses will be considered same for estimation of similarity value. Therefore, there is a need to estimate allele dosage also. The present results offer an avenue of some closely related wild species with *S. tuberosum* for widening the genetic base of the future potato cultivars.

This is the first report of using the genome specific primers in potato. Study indicates that use of these markers can clearly distinguish different cultivated, semi-cultivated and wild species. Generation of the genetic profiles using the genome specific primers helps in documentation of ownership and protection of intellectual property rights along with other molecular marker systems (RAPD, AFLP, and SSR). The present study also clearly elucidated the genetic relatedness among the genotypes, which helps in selection of the diverse genotypes for genetic improvement of potato using unexploited wild species. It will also facilitate the construction of library of introgression lines (IL's) of the wild species in genetic background of cultivated potato which in turn would help in genetic study of the agronomically important traits found in the wild potato species.

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Inter Simple Sequence Repeat (ISSR) markers for identification of germplasm and diversity analysis in coconut (*Cocos nucifera* L.)

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ABSTRACT

Inter Simple Sequence Repeat (ISSR) is a powerful molecular marker technique used for variety of purposes like genetic diversity analysis, phylogeny information, germplasm identification, gene tagging etc. In the present paper, 33 coconut (*Cocos nucifera* L.) germplasm representing different geographical regions have been fingerprinted with highly polymorphic ISSR markers. Ten polymorphic primers targeting the microsatellite regions were used to amplify the coconut germplasms. Ten primer produced 104 polymorphic markers with 87 percent polymorphism. Cluster analysis was carried out among 33 accessions by UPGMA using the software POPGENE ver.1.32. The dendrogram revealed five clusters. Based on the fingerprint data germplasm specific markers were identified using the primers UBC 810, UBC 915, UBC 823, UBC 824 and UBC 855. The marker UBC 815_{1673} was specific to Panama Tall (PNT) and Saint Vincent Tall (STVT). The marker UBC 854_{573} was specific to Nuwehnug Tall (NWHT). ISSR markers were proved to be useful for generating fingerprints and for deriving genetic relationships in coconut.

Keywords: Coconut, ISSR markers, germplasm, specific markers

INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is one of the major perennial oil crops of tropics providing the basis for food and industrial products in many developing countries. So far, germplasm accessions have been characterized and identified using morphological and nut characteristic such as plant stature as tall/dwarf, crown shape, leaf characters, nut shape, nut color etc. These characteristics are still continued to be used in the germplasm bank at Central Plantation crops Research Institute (CPCRI), India. But, the task of unambiguously identifying the new accessions will be difficult by the use of conventional characteristics alone. It is apparent that use of molecular markers would solve the problem of providing unique DNA profiles for the germplasms, with aid of morphological characteristics.

Molecular markers like RFLPs (Lebrun *et al*., 1998), RAPD (Everard, 1999, Upadhyay *et al*., 2004), AFLP (Perera *et al*., 1998), SSR (Perera *et al*., 2000, 2003. Rajesh *et al*., 2008) and ISSR (Manimekalai, 2005) have been reported in coconut for genetic diversity estimation. Cultivar specific markers from retrotransposon-based insertional polymorphism (RBIP) based on the long terminal repeat (LTR) sequences have been developed for Japnese Pear (Kim *et al.*, 2012) and for the analysis of diversity (Kalendar *et al*., 2011). The cost involved in the development of SSR markers necessitates the use of Inter Simple Sequence Repeat (ISSR) markers. ISSR PCR is a technique that overcomes the problems like low reproducibility of RAPD, high cost of AFLP, and the need to know the flanking sequences to develop species specific primers for SSR polymorphism (Goodwin *et al*., 1997). ISSRs have high reproducibility possibly due to the use of longer primers (16-25 mers) as compared to RAPD primers (10 mers) which permit the subsequent use of high annealing temperature (45-60 °C) leading to higher stringency. Because of high polymorphism and reproducibility, it seemed likely that this maker would be useful for germplasm identification in coconut. The objective of the work reported here was to assess the ability of ISSR to distinguish the coconut accessions and to discern genetic relationships among a set of coconut germplasm.

MATERIALS AND METHODS

DNA was extracted from 2g of fresh leaf material from 132 individuals of 33 germplasm (4 individuals per accession) using Plant DNA extraction kit (In vitrogen) as per the manufacturer's instructions (Table 1). Amplification reactions were carried out as per the standardized protocol (Manimekalai, 2005). ISSR primer sequences and primers were supplied by University of British Columbia, Canada. Ten highly polymorphic primers were used to amplify the DNA. These primers were selected based on preliminary primer screening of 40 ISSR primers and then selected highly polymorphic 10 primers. Amplifications were

performed in a Thermo Cycler (MJ Research Inc.) programmed for an initial denaturation at 94 °C for 5 min., 40 cycles of 1 min., denaturation at 94 °C, 1 min., annealing at 50 ºC and 2 min. extension at 72 ºC and a final extension of 7 min. at 72 ºC. The PCR products were electrophoresed in 1.80 % agarose gels.

Only the clear, unambiguous and reproducible bands were considered for scoring. Each band was considered to represent a single locus. Data were scored as 1 for the presence and 0 for the absence of a DNA band of each accession. DNA band size was estimated by comparing the DNA bands with a 1 Kb and 100 bp DNA ladder or lambda DNA Eco RI / Hind III double digest (MBI Fermentas, Lithuania). Binary data were analyzed using the software POPGENE version 1.32 (Yeh and Boyle, 1999). The dendrogram was constructed using UPGMA based on Nei's unbiased genetic distance in the software Phylip 3.5. The markers produced by the individual primer were labeled by suffixing the molecular weight of the respective band.

RESULTS AND DISCUSSION

Ten highly polymorphic ISSR primers detected a total of 120 markers across 33 coconut germplasms, out of which 104 were polymorphic. The number of markers per primer varied from nine (UBC 854) to 17 (UBC 854), with a mean of 12 markers per primer. The number of polymorphic markers for each primer varied from seven (UBC 835) to 15 (UBC 854 and UBC 855) with a mean of 10.4. The product size ranged from 206 bp (UBC 834) to 2618 bp (UBC 815) (Table 2). Fingerprint pattern produced by the primer UBC 889 (Fig. 2).

ISSR primers produced specific markers for germplasm. The primer UBC 810 produced three markers (Table 3).

Table 1: Details of coconut germplasm accessions

No.	Accession	Code	Geographic region
1.	Kong Thienyong Tall	KTYT	Southeast Asia
2.	Straight Settlement Green Tall	SSGT	Southeast Asia
3.	Straight Settlement Apricot Tall	SSAT	Southeast Asia
4.	Philippines Kalambahim Tall	PKBT	Southeast Asia
5.	Laguna Tall	LAGT	Southeast Asia
6.	Philippines Palawan Tall	PPWT	Southeast Asia
7.	Philippines Dalig Tall	PDLT	Southeast Asia
8.	San Roman Tall	SNRT	Southeast Asia
9.	Markham Valley Tall	MVT	South Pacific
10.	Nufella Tall	NUFT	South Pacific
11.	Nugili Tall	NUGT	South Pacific
12.	Nuwallis Tall	NUWT	South Pacific
13.	Nu Quamen Tall	NUQT	South Pacific
14.	Kupien Tall	NHKT	South Pacific
15.	Nuwehnug Tall	NWHT	South Pacific
16.	Lifou Tall	LFT	South Pacific
17.	British Solomon Island Tall	BSIT	South Pacific
18.	Jamaica Tall	JMT	Atlantic
19.	Saint Vincent Tall	STVT	Atlantic
20.	Panama Tall	PNT	America
21.	Nigerian Tall	NIT	Africa
22.	Kaithathali Tall	KAIT	South Asia
23.	Indian Spicata	WCT01	South Asia
24.	Indian East Coast Tall	ECT	South Asia
25.	Verrikobbari Tall	VKBT	South Asia
26.	Nadora Tall	NDRT	South Asia
27.	Nicobar Tall	NICT01	South Asia
28.	Hazari Tall	HZT	South Asia
29.	Navassi Tall	NAVT	South Asia
30.	Niuleka Dwarf	NLAD	South Pacific
31.	King coconut	RTB04	South Asia
32.	Laccadive Dwarf	LCOD	South Asia
33.	Chowghat Orange Dwarf	COD	South Asia

The marker UBC 855_{2155} was unique to dwarf coconuts studied here and two of the South Pacific coconut populations, Nufella Tall (NUFT) and Nuwallis Tall (NUWT). The marker UBC 815_{1673} was specific to Panama Tall (PNT) and Saint Vincent Tall (STVT). UBC 889_{914} was specific to Philippines Palawan Tall (PPWT), San Ramon Tall (SNRT), Nuwallis Tall (NUWT), Saint Vincent Tall (STVT), of Nicobar Tall (NICT 01), Kaitha4thali Tall (KAIT) and Hazari Tall (HZT). This is the first report of use of ISSR markers in germplasm identification in coconut, however they are available in other crops. In cashew ISSR fingerprints have been developed for the germplasm identification (Archak *et al*., 2003)

Dendrogram was constructed based on average genetic distance among 33 coconut germplasming (Fig. 1) by UPGMA method. The dendrogram revealed five clusters. Cluster I consisted of germplasm belonging to the South East Asia (KTYT, SSGT, SSAT, PKBT, LAGT, PPWT, SNRT) and South Pacific region (MVT, NUWT, NUQT, LFT) . Dwarf and intermediate population (LCOD, NLAD, RTB 04) and Indian Spicata (WCT 01) also clustered within cluster I. Indian Spicata (WCT 01) which is characterized by a single spike, without any spikelets that bears many female flowers is generally considered a variant of West Coast Tall (WCT) in India. It showed clear proximity to South East Asia coconut in this study. Previous reports (Manimekalai and Nagarajan, 2006) also suggested the clustering of Indian Spicata coconuts with South East Asian accessions. The dwarf coconuts clustering with the South East Asia coconuts are in agreement with the earlier report

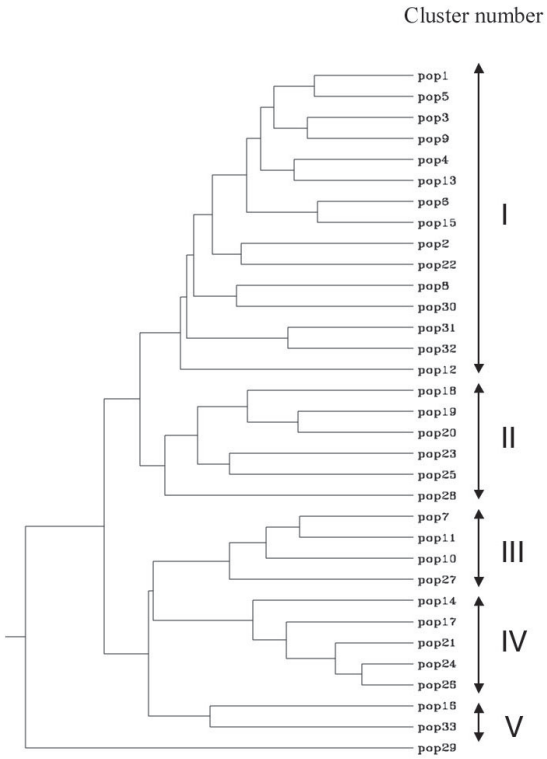
Table 2: Details of primers used and markers produced in coconut germplasms

S.No.	Primer	Sequence	Total markers (No.)	Polymor phic markers (No.)	Product size (bp)
1.	UBC815	CTCTCTCTCTCTCTCTG	13	13	2618-554
2.	UBC834	AGAGAGAGAGAGAGAGYT	12	10	1252-206
3.	UBC841	GAGAGAGAGAGAGAGAYC	10	8	2316-698
4.	UBC810	GAGAGAGAGAGAGAGAT	9	8	2443-879
5.	UBC824	TCTCTCTCTCTCTCTCG	10	9	2455-506
6.	UBC835	AGAGAGAGAGAGAGAGYC	10	7	2375-514
7.	UBC854	TCTCTCTCTCTCTCTCRG	17	15	1545-299
8.	UBC855	ACACACACACACACACYT	15	15	2459-606
9.	UBC889	DBDACACACACACACAC	14	10	2069-290
10.	UBC823	TCTCTCTCTCTCTCTCC	10	9	2287-877
	Total		120	104	
	Mean		12.0	10.4	

Y= (C, T)
D= (A, G, T)
R= (A, G)
B=(C,G,T) ie not A

Table 3: List of ISSR markers identified for the coconut germplasms

S. No.	ISSR marker	Germplasms identified
1.	UBC 889_{914}	Philippines Palawan Tall (PPWT), San Roman Tall (SNRT), Nuwallis Tall (NUWT), Saint Vincent Tall (STVT), Kaithathali Tall (KAIT), Nicobar Tall (NICT 01) and Hazari Tall (HZT)
2.	UBC 810_{1543}	Hazari Tall (HZT), Niu Leka Dwarf (NLAD) and King coconut (RTB 04)
3.	UBC 810_{879}	Kupien Tall (NHKT)
4.	UBC 810_{674}	Straight Settlement Apricot Tall (SSAT) and Hazari Tall (HZT)
5.	UBC 815_{1673}	Saint Vincent Tall (STVT) and Panama Tall (PNT)
6.	UBC 823_{1081}	Straight Settlement Apricot (SSAT), Nuwallis Tall (NUWT) and Navassi Tall (NAVT)
7.	UBC 824_{1008}	Jamaica Tall (JMT), Panama Tall (PNT), Nigerian Tall (NIT) and Nadora Tall (NDRT)
8.	UBC 824_{939}	Hazari Tall (HZT), San Roman Tall (SNRT), Markham Valley Tall (MVT), Philippines Kalambahim Tall (PKBT), Nu Quamen Tall (NUQT), Jamaica Tall (JMT), Panama Tall (PNT), Saint Vincent Tall (STVT), Nigerian Tall (NIT) and Chowghat Orange Dwarf (COD)
9.	UBC 854_{952}	Straight Settlement Apricot Tall (SSAT)
10.	UBC 854_{573}	Nuwehnug Tall (NWHT)
11.	UBC 855_{2155}	Nufella Tall (NUFT), Nuwallis Tall (NUWT), King coconut (RTB 04), Laccadive Dwarf (LCOD) and Chowghat Orange Dwarf (COD)



POP1=KTYT; POP2=SSGT; POP3=SSAT; POP4=PKBT; POP5=LAGT; POP6=PPWT; POP7=PDLT;POP8=SNRT; POP9=MVT; POP10=NUFT; POP11=NUGT; POP12=NUWT; POP13=NUQT; POP14=NHKT; POP15=LFT; POP16=BSIT; POP17=JMT; POP18=STVT; POP19=PNT POP 20=NIT; POP21=KAIT; POP22=WCT01; POP23=ECT; POP24=VKBT; POP25=NDRT; POP26=NICT01; POP27=NWHT POP28=HZT; POP29=NAVT POP30=NLAD; POP31=RTB04; POP32=LCOD; POP33=COD)

Fig. 1: Dendrogram of 33 coconut germplasm based on 120 ISSR markers constructed using UPGMA based on Nei's unbiased genetic distance in the software Phylip 3.5

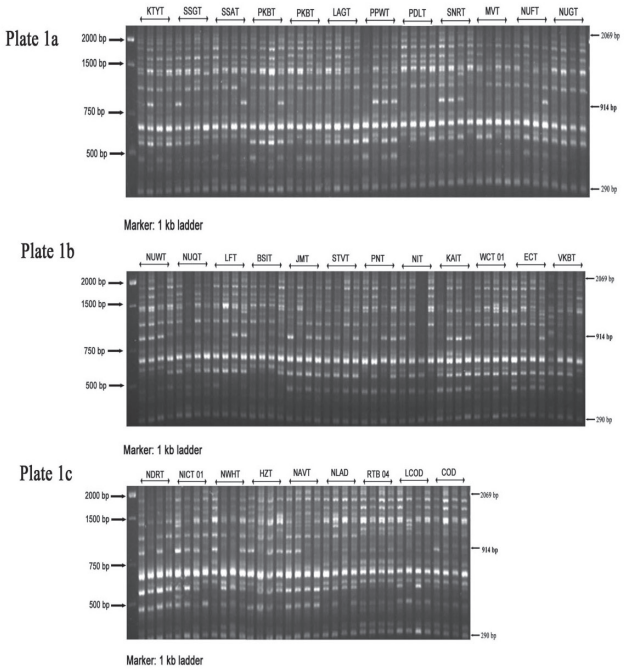


Fig. 2: UBC 889 ISSR profile of coconut accessions showing specific markers

(Perera *et al*., 2003). Cluster II comprised accessions belonging to South Asian (ECT, HZT, NDRT), Atlantic and American populations (PNT, STVT) and African populations (NIT). Generally, a grouping of population based on the geographic origin was observed, except in Philippines Dalig Tall (PDLT), Kaithathali Tall (KAIT), Verrikobari Tall (VKBT), Nicobar Tall (NICT 01) and Indian Spicata (WCT 01). The clustering pattern of coconut population obtained here using ISSR markers is comparable with that of clusters produced by RAPD markers (Lebrun *et al*., 1998). The accession Navassi tall (NAVT) occupied separate position in the dendrogram, even though this accession belongs to the South Asia region, It reveals that the NAVT has variability among South Asian accessions. Based on our study, ISSR markers are found to be useful in generating fingerprints for coconuts and to infer genetic relationships among the coconut accessions. Moreover, ISSR technique is robust compared to RAPD, technically less demanding than AFLP and less complex than SSR. With the less infrastructure facility this technique could be performed. Hence, ISSR markers have potential in genotypic studies in coconut. Barcode for the Palmae family species are being developed (Jeanson *et al*., 2011) based on *matK, rbcL* and nrITS2 for species identification, however, for germplasm identification, highly polymorphic markers are useful than the barcodes.

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Chromatographic fingerprinting and estimation of organic acids in selected *Garcinia* species

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Abstract

Garcinia is more valued as medicinal crop because of the presence of Hydroxy citric acid (HCA). Studies on the presence of other acids like malic acid, citric acid, oxalic acid and tartaric acid will improve the importance of the crop. HPLC chromatographic studies indicated that the *Garcinia* species showed a great variation in the number of peaks and their relative area. Among the Western Ghats species (*G. gummi-gutta, G. tinctoria,G. indica and G. Subelliptica)* along with the Malayan species *G. mangostana*, the peak corresponding to HCA (Peak No.4) had the largest area while in case of the species from NE Himalayas, the Peak3 corresponding to malic acid was the largest. In case of *G. tinctoria*, the largest peak was for citric acid with small peak for HCA. In all the species, the peaks corresponding to tartaric acid, oxalic acid and ascorbic acid were small. Total acids obtained were highest in *G. kidya/Cowa* 28% followed by *G. gummi-gutta* and *G. pedunculata* (15.8%) while it is less in *G. mangostana* (4.5%)

Key words: *Garcinia HCA, G. gummi-gutta, G. tinctoria, G. indica and G. Subelliptica, G. mangostana*, hydroxy citric Acid, malic acid, citric acid, oxalic acid and tartaric acid.

INTRODUCTION

Garcinia is a plant genus of the family Clusiaceae, native to Asia, Australia, tropical and southern Africa, and Polynesia, includes about 300 species. About 35 species are found in India among which 17 are endemic. Many species are threatened due to habitat destruction (Cheek, 2004). It is an important medicinal crop due to the presence of many beneficial compounds like phenols and acids in the fruit rinds. Utpala *et al.* (2010) quantified the presence of HCA in the fruit rind of 8 species of Western Ghats & North East Himalayas. The present work focuses on the identification and quantification of organic acids present in the dried rinds of 8 different species from Western Ghats and N.E. Himalayas.

Organic acids are the product of metabolism and stored in the vacuoles of cell, which are colourless, non-volatile and water soluble compounds and can be easily detected using acid indicators like bromocresol green. Common plant organic acids are malic, citric, tartaric, oxalic and ascorbic acids. Acidity plays an important part in the perception of fruit quality. It affects the sweetness of fruit by masking the taste of sugars (Lyon *et al.*, 1993). A chemical fingerprint obtained by hyphenated chromatography, out of question, will become the primary tool for quality control of herbal medicines (Lazarowych and Pekos, 1998). Organic acids can penetrate the bacteria cell wall and disrupt the normal physiology of certain types of bacteria. It lowers pH of cytoplasm and increases osmotic potential of cell, making the cells stop functioning and get damaged. Hence acids like citric acid, tartaric acid and lactic acid is used as food preservatives and anti-microbial solutions (Brul and Coote, 1999). Organic acids are also used to cure gastro intestinal tract infections and control gut microflora. They are provided in cattle and poultry feeds to improve digestive health of animals without any adverse health-ethic impact and so can be used in foods for babies and ill-people (Dibner and Butin, 2002). In food and medicine industries, addition of organic acid increases shelf-life of foods and drugs, and reduces chances of microbial contamination. They are preferred over benzoic acid derivatives as organic acids are non-toxic and highly water soluble (Patanen and Mroz, 1999). Organic acids provide flavor to food. According to Silva *et al.* (2004), organic acids may have a protective effect against multiple diseases due to their antioxidant activity.

Considering the importance of organic acids and the *Garcinia* species in medicine and food, the present studies were conducted. This is the first report of such a study in *Garcinia*.

MATERIALS AND METHODS

Dried fruit rinds of 8 species namely *G. gummi-gutta, G. tinctoria, G. indica, G. mangostana* and *G. subelliptica* of W. Ghats and *G. pedunculata* (Bor thekera), *G. lancifolia* (Rupohi thekera) and *G. kidya* (Kuji thekera) of N.E Himalayas were taken for the experiment. 5 g of finely chopped rinds were refluxed with 20 ml de-ionized water for 1 hour, filtered and transferred extract into 100 ml standard flask. Repeated the extraction with fresh distilled water and pooled up the extracts. Final volume was then made up to 100ml with de-ionized water. Total acidity was estimated by acid-base titration method against 0.075M NaOH and phenolphthalein as the indicator. Detection of organic acids was performed by ascending paper chromatography using Whatman No.1 paper of about 20 × 5 cm dimension. A proportionate mixture of n-butanol, formic acid and water was taken as the solvent (5:1:5). After completion, the paper was dried and sprayed with the indicator and re-dried. Indicator solution was 1% solution of bromocresol green which was then made alkaline using sodium hydroxide to obtain deep blue colouration. Rf values for each organic acids were calculated and compared with that of standard acids. Estimation of organic acids was performed using Reverse Phase HPLC. 50mM phosphate solution was taken as mobile phase for HPLC whose pH was adjusted to 2.1 using ortho-phosphoric acid. pH 2.1 was selected due to better resolution. Standard solutions for various organic acids were prepared separately with concentrations of malic acid (7000 mg/l), citric acid (5000 mg/l), oxalic acid (1000 mg/l), ascorbic acid (1000 mg/l), acetic acid (750 mg/l), tartaric acid (5000 mg/l) and succinic acid (5000 mg/l) in de-ionized water and then filtered. Standards are stored at 4 °C. Succinic acid is used as internal standard and response factor were calculated for each acids.

HPLC instrument and conditions

HPLC system comprising of Shimadzu LC-10AT pump, SPD-10A VP UV-VIS detector, SCL-10A VP controller and C-18 reversed phase column was used for analysis. Analysis were made at 214nm wavelength at a flow rate of 1.00ml per minute.

RESULTS AND DISCUSSION

The paper chromatograms are given in Fig.1. The HPLC chromatograms are given in Fig. 2 a&b. In HPLC, the conversion factors for each organic acids were calculated using the peak area information and the concentration of the concerned organic acids as well as the internal standard (succinic acid). The species showed a great variation in the number of peaks and their relative area. Among the Western Ghats species (*G. gummi-gutta, G. tinctoria, G. indica and G. subelliptica)* along with the Malayan species *G.mangostana*, the peak corresponding to HCA (Peak No.4) had the largest area while in case of the species from NE Himalayas, the Peak 3 corresponding to malic acid was the largest. In case of *G. tinctoria*, the largest peak was for citric acid with small peak for HCA. In all the species, the peaks corresponding to tartaric acid, oxalic acid and ascorbic acid were small.

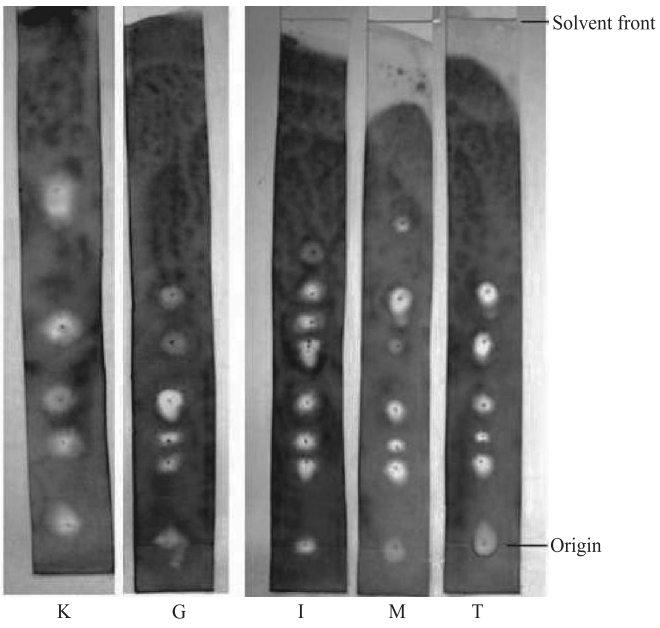


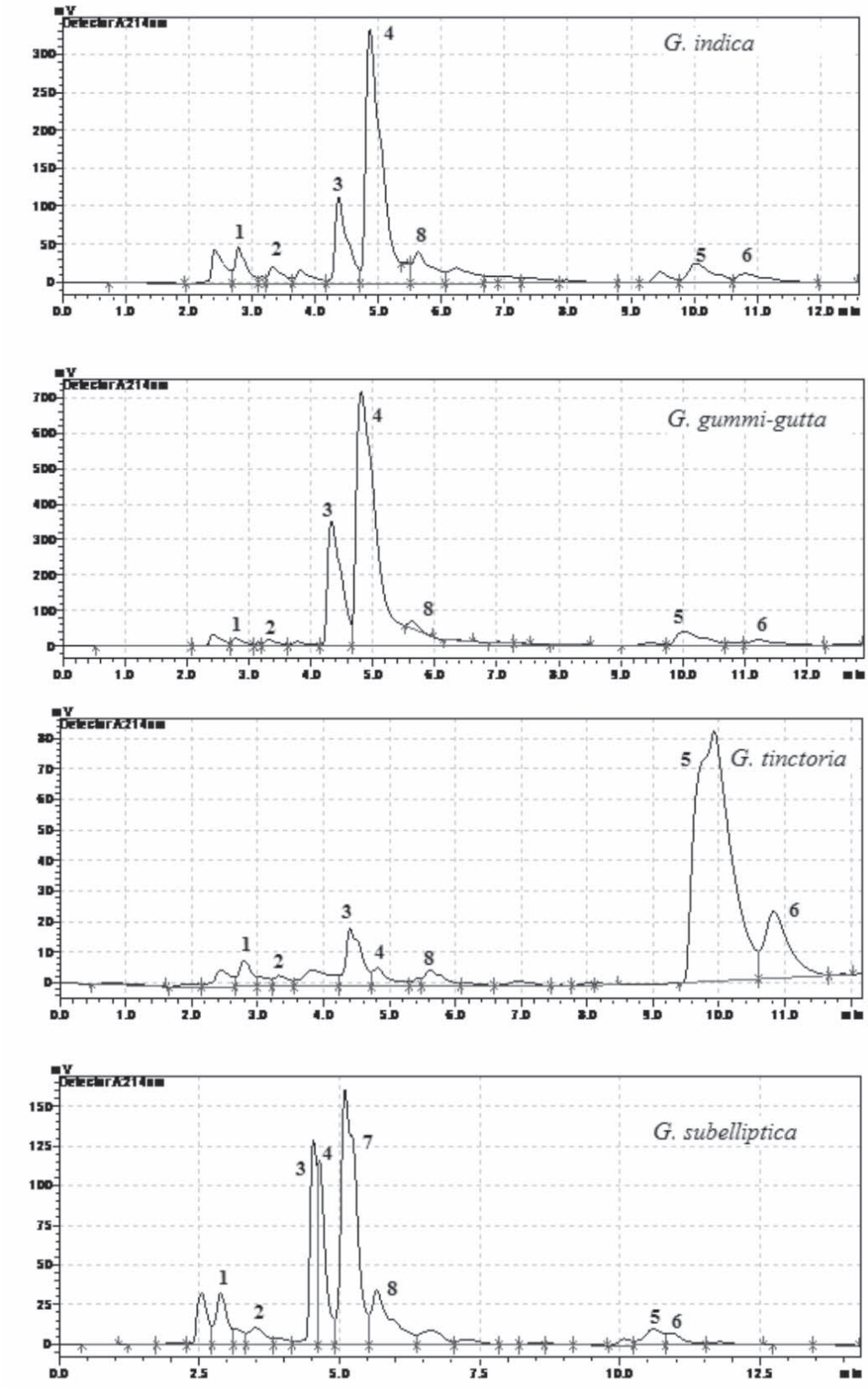
Fig. 1: Paper Chromatograms for organic acid detection

Table 1: Retention factor of organic acids in Garcinia samples:

Sample	No.of spots	Rf values
G. gummigutta	5	0.15, 0.20, 0.26, 0.37, 0.45
G. indica	7	0.16, 0.22, 0.26, 0.36, 0.40, 0.45, 0.67
G. tinctoria	5	0.15, 0.20, 0.25, 0.37, 0.45
G. mangostana	6	0.14, 0.22, 0.26, 0.37, 0.46, 0.59
G. kidya	5	0.13, 0.20, 0.24, 0.38, 0.45
G. pedunculata	6	0.15, 0.21, 0.25, 0.36, 0.45, 0.59
G. lancifolia	6	0.16, 0.21, 0.24, 0.38, 0.44, 0.54
G. subelliptica	6	0.14, 0.20, 0.24, 0.36, 0.45, 0.59

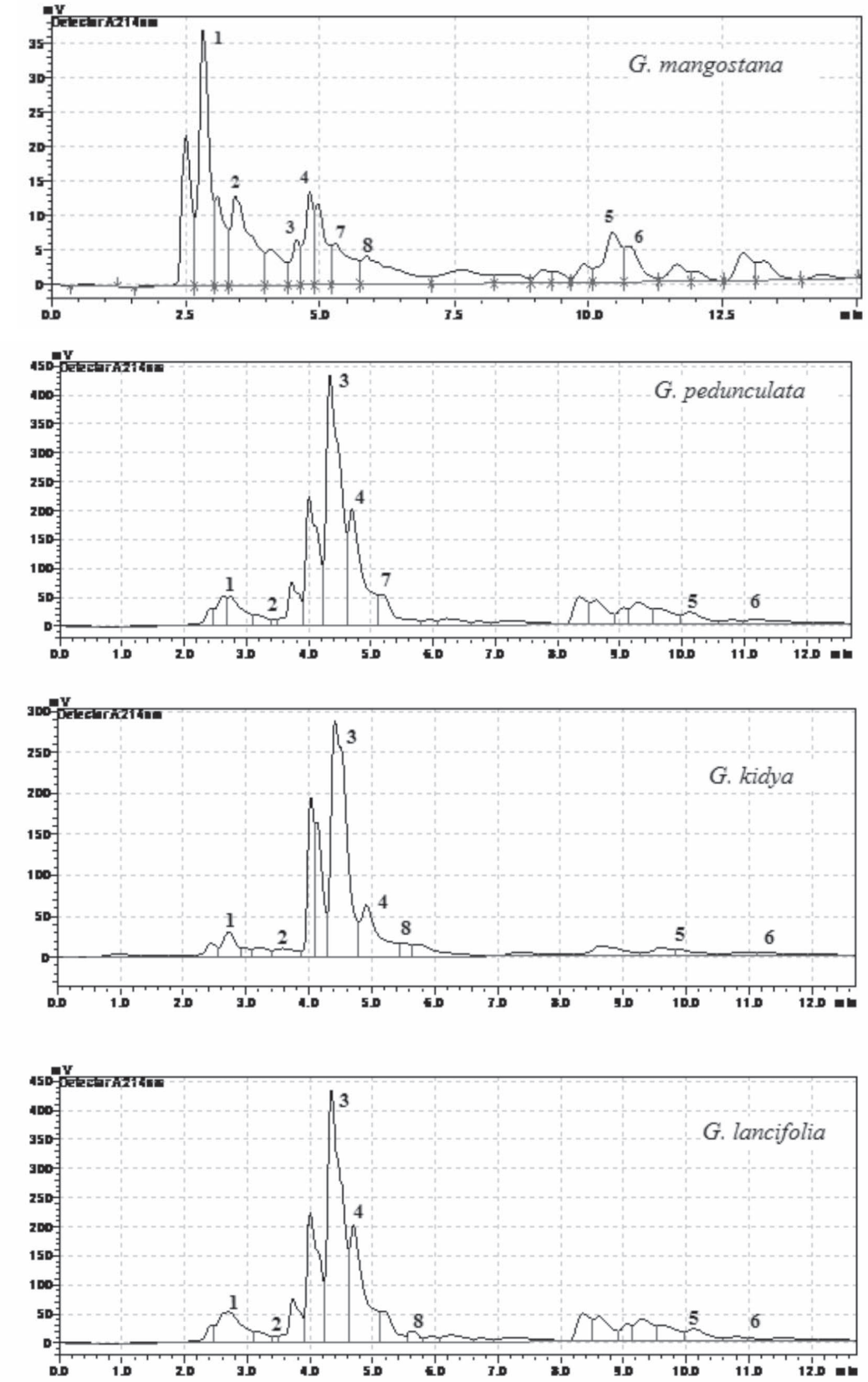
The Rf values of standard acids were found to be oxalic acid (0.14), tartaric acid (0.21), malic acid (0.45), citric acid (0.38), hydroxycitric acid (0.24) and ascorbic acid (0.60). These values were found to be in agreement with that reported by Harborne (2005) with deviation of +- 0.01 to 0.07.

Total acids obtained were highest in *G. kidya/Cowa* (28%) followed by *G. gummi-gutta* and *G. pedunculata.* (15.8%) while it is less in *G. mangostana* (4.5%) (Table 2). Common



Peaks 1 to 8 refer to Oxalic acid, tartaric acid, malic acid, Hydroxycitric acid, Citric acid, succinic acid, ascorbic acid and acetic acid respectively.

Fig. 2a: HPLC chromatograms for *Garcinia* organic acid profiling



Peaks 1 to 8 refer to Oxalic acid, tartaric acid, malic acid, Hydroxycitric acid, Citric acid, succinic acid, ascorbic acid and acetic acid respectively.

Fig. 2b: HPLC chromatograms for *Garcinia* organic acid profiling

Table 2: Organic Acid profile of *Garcinia* species

Sample	Total acidity (g %)	HCA (g %)	Malic acid (g %)	Oxalic acid (g %)	Citric acid (g %)	Tartaric acid (g %)	Acetic acid (g %)	Ascorbic acid (g %)
G. gummi-gutta	15.81	10.48	4.62	0.18	0.62	0.11	0.07	trace
G. indica	8.11	6.13	1.67	0.63	0.79	0.51	0.31	trace
G. mangostana	4.49	0.26	0.44	0.73	1.42	1.66	0.26	0.41
G. tinctoria	8.95	0.05	0.31	0.37	6.00	0.10	0.04	trace
G. subelliptica	9.16	1.16	3.77	0.92	0.81	0.88	1.22	4.61
G. kidya/Cowa	28.00	8.97	14.32	0.60	1.35	1.80	0.23	trace
G. lancifolia	12.17	1.93	10.02	1.70	1.45	0.07	0.14	trace
G. pedunculata	15.92	1.02	8.95	0.51	1.30	0.12	trace	1.54

plant organic acids are malic, citric, tartaric and ascorbic acids. Acidity plays an important role in the perception of fruit quality. It imparts not only the sour taste to the fruit, but also masks the sugars in the ripened fruit (Lyon *et al.*, 1993).

Hydroxycitric acid (HCA), 1,2-dihydroxypropane-1,2,3-tricarboxylic acid is well known acid of *Garcinia* present in all the species which is more than 1%, except in *G. tinctoria* and *G. mangostana*. *G. gummi-gutta* which are having highest 10.48%. *G. kidya/Cowa* and *G. indica* is also having mordarately high 8.97 and 6.13% respectively. Hydroxycitric acid is a potent anti-obesity compound and reduces lipid and sugar levels in blood. Sullivan & Triscari (1977) reported that (-) HCA lowered body fat level with no loss of body protein or lean mass in test animals that had been experimentally made obese. Thus as natural product of *Garcinia* the hydroxycitric acid is working wonders in reducing weight and obesity. HCA is a potent metabolic regulator of obesity and it also lowers blood lipids such as cholesterol and triglycerides. Thus, it is increasingly becoming important industrially, commercially and medicinally

Malic Acid occurs naturally almost in all fruits. It is generated during fruit metabolism. Malic acid is used as an ingredient in skin preparations (combined with benzoic acid and salicylic acid) for the removal of dead skin from ulcers, burns, and wounds. It is also an ingredient in artificial saliva preparations. Malic acid is also present in good concentration in *G. kidya/Cowa*, (14.32%), G. lancifolia (10.02%) and in *G. pedunculata* (8.95%). It is interesting to note that all Himalayan samples are having more percentage of malic acid than Western Ghats samples. G. lancifolia is known as *Rupohi thekara* in Assam and the extract is used for skin beautification. Malic acid is added to many foods and candies to give them a more tart taste. Fruity-tasting candies and confections rely on malic acid as a flavoring agent, as do some sodas. Malic acid can be used to preserve certain flavors, or enhance flavors in processed foods that lose some of their natural flavoring during the processing, like canned fruits. A study conducted in 2009 published by a group of doctors in the "Journal of Clinical Biochemistry and Nutrition" suggested that malic acid is beneficial in lowering blood pressure. The Food and Drug Administration identifies malic acid as being an effective pain reliever for certain conditions, like the pain sustained from an ischemic reperfusion injury. It can also act as an anti-inflammatory agent (www.livingstrong.com).

Citric acid is nominal in all the species except in *tinctoria* where HCA is less and citric acid is high. *G. mangostana* is have considerably high (1.42%) citric acid. All Himalayan species have more than 1% citric acid, which is a weak organic acid, sold in a dry powdered form and is commonly sold in markets and groceries as "sour salt", due to its physical resemblance to table salt. It has use in culinary applications where an acid is needed for either its chemical properties or for its sour flavor, but a dry ingredient is needed and additional flavors are unwanted (e.g., instead of vinegar or lemon juice). A solution with a 6% concentration of citric acid will remove hard water stains from glass without scrubbing. In industry, it is used to dissolve rust from steel. It can be used in shampoo to wash out wax and coloring from the hair. (Frank, 2005).

Oxalic acid and oxalates are present in many plants, including tea and cocoa. Within the sub-group of 'weak acids', oxalic acid is relatively strong. Oxalic acid is a chemical that can be a dangerous in high-doses but not in moderate levels. In *G. indica*, oxalic acid percentage is comparatively high (0.63%). The Himalayan sample *G. lancifolia* is having good amount of *oxalic* acid (1.7%). It is also used for juice preparation. Oxalic acid has many uses, but it is widely used as a wood bleach because it removes stains without removing the natural color of the wood. It was identified as the acid which kills cancer cells without harming normal cells (www.youtube.com/watch).

Ascorbic acid is present only in traces in Garcinia species except *G. Subelliptica* and *G. pedunculata*. Ascorbic acid which is also known as vitamin C is a good antioxidant and anti-ageing chemical. It is an important co-factor for

several enzymes and essential for collagen synthesis. It is used to prevent cold and ulcers. It is known to enhance immunity. It is used in cosmetic industries for reducing ageing symptoms and enhancing wound healing (Garret and Grisham, 2005). Sometimes ascorbic acid is put on their skin to protect it against the sun, pollutants, and other environmental hazards.

Tartaric acid is high in *G. kydia* and at moderate level in *G. pedunculata*. It occurs in traces in other species. Tartaric acid is among the fruit acids used as a flavoring ingredient in soft drinks. It also adds tartness in foods. Tartaric acid is used in the tanning of leather. As a di-acid and a di-alcohol, it can be used in polymeric products, including lacquers. Additionally, it is used in textile printing and blue printing applications (http://www.ehow.com/about_5349916_use-tartaric-acid.html)

Acidity plays an important part in the perception of fruit quality. It affects not only the sour taste of the fruit but also sweetness, by masking the taste of sugars. In the recent years *Garcinia* is more valued as medicinal crop because of the presence of HCA. Studies on the presence of other acids like malic acid, citric acid, oxalic acid and tartaric acid will improve the importance of the crop. HPLC chromatographic studies indicated that the *Garcinia* species showed a great variation in the number of peaks and their relative areas. Among the Western Ghats species (*G. gummi-gutta* **,** *G. tinctoria*,*G. indica and G. Subelliptica)* along with the Malayan species *G. mangostana*, the peak corresponding to HCA (Peak No.4) had the largest area while in case of the species from NE Himalayas, the Peak 3 corresponding to malic acid was the largest. In case of *G. tinctoria*, the largest peak was for citric acid with small peak for HCA. In all the species, the peaks corresponding to tartaric acid, oxalic acid and ascorbic acid were small. Total acids obtained were highest in *G. kidya/Cowa* (28%) followed by *G. gummi-gutta* and *G. pedunculata* (15.8%) while it is less in *G. mangostana* (4.5%).

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Effect of sub lethal dose of insecticides on electrophysiological and behavioral response of *Bracon brevicornis* Wesmael (Braconidae : Hymenoptera) a parasitoid of coconut black headed caterpillar, *Opisina arenosella* Walker (Oecophoridae : Lepidoptera)

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ABSTRACT

Bracon brevicornis Wesmael (Braconidae : Hymenoptera) is an effective parasitoid against coconut black headed caterpillar, *Opisina arenosella* Walker (Oecophoridae : Lepidoptera) a major pest on coconut. During outbreaks, chemical insecticides and bioagents are used in tandem to manage *O. arenosella*. Traditionally the impact of pesticides on beneficial arthropods was assessed by determining acute toxicity. In addition to direct mortality caused by insecticides, the sublethal effect on sensory physiology and behavior of parasitoids must be considered for complete analysis of their impact. The present study aims to investigate the impact of sub lethal dose of insecticides on *B. brevicornis*. In olfactory assay, the starved parasitoids oriented to food source, whilst the fed parasitoids responded more to host volatiles. In electrophysiological assay, the antennal responses of *B. brevicornis* to host volatiles were higher in female. Among the volatiles tested, the host frass and host haemolymph was most preferred by both sexes. Exposure of *B. brevicornis* to sublethal dose of imidacloprid, malathion and carbaryl (LD_{20}) altered the orientation of *B. brevicornis* to host volatiles.

Key words: Behavior, *Bracon brevicornis*, EAG, insecticide, *Opisina arenosella.*

INTRODUCTION

Coconut (*Cocos nucifera* Linn.), a perennial crop is attacked by an array of pests round the year. Among the key pests, coconut black headed caterpillar (*Opisina arenosella* Walker) (Oecophoridae: Lepidoptera) is a serious one. The larval stage of the *O. arenosella* feeds on the parenchymatous tissues on the undersurface of the leaflets and constructs galleries of silk and frass. Under favorable conditions the pest multiplies rapidly and devastates the leaf lamina (Venkatesan *et al.,* 2003). In the year following severe outbreak, there is a crop loss of 45.4% in terms of nut yield. It takes around four years for the infested palms to regain their normal yield (Chandrika Mohan *et al*., 2010).

O. arenosella is managed by spraying dichlorvos @ 0.02% (Nair *et al*., 1998). In addition to spraying chemical pesticides, the ecto parasitoids are released to target *O. arenosella*. The success of inundative release of *Bracon brevicornis* Wesmael (Braconidae: Hymenoptera) to manage *O. arenosella* was reported by Venkatesan *et al*., (2009). The parasitoids released in the field are exposed to insecticides through droplets or residues on crop foliage or when feeding on contaminated water droplets and nectar. There are no studies on the behavioral effects of insecticide on *B. brevicornis*. The detection of chemical stimuli and its association to adapted behavior is entirely dependent on nervous transmission (Komez *et al*., 2001). Considering the above, the present study was taken up to study the response of *B. brevicornis* to host volatiles when exposed to sub lethal dose of insecticides.

MATERIALS AND METHODS

The study was conducted at Central Plantation Crops Research Institute, Kasaragod, India (Latitude – $12^{0}3$ 0'N; Longitude 75^{0} 00'E). *Bracon brevicornis* was mass multiplied on alternate host rice moth, *Corcyra cephalonica* larvae. A pair of freshly eclosed parasitoid (male and female) was introduced in a tube (7.5 × 2.5cm) for mating. After a pre-oviposition period of 6 days, a last instar larva of *C. cephalonica* was provided in the vial. The paralyzed larva was removed and a new larva was introduced into the vial. The life cycle of the parasitoid was completed in 10-14 days. The cultures were maintained at 25 ^{o}C and 65 – 70 % RH with a constant dim light illumination.

Olfactory assay

'Y' tube made of clear glass having two arms of 10 cm

length and stem of 20 cm length with an internal diameter of 3 cm was used in the assay. The odor source was placed in one arm and control in other arm. The adult parasitoids were released in the stem of the 'Y' tube and secured with muslin cloth. The airflow was passed into the arms through activated charcoal cartridge @ 30 ml / min. Constant airflow was maintained in both arms. A fluorescent bulb was placed in front of the Y arms. The parasitoids (0 -12 hrs old) fed / starved for 24 hrs were used for the assay. The choice of the parasitoids was assessed from the tracks of the images captured. The parasitoid movements and their choice of the arm was recorded by the JVC TK C 1380 CCD camera, which in turn was connected to Pinnacle video capturing card in the CPU. The data was captured at 640 × 480 resolution at the rate of 30 frames per second and stored in MPEG format. The MPEG video clips were converted into AVI format in 'true color' image type and 'indeo5' video compression. The program was implemented using the functions of Image Processing Toolbox in MATLAB® Release14. The MATLAB script was run with a graphical user interface on a Dell Optiplex GX270, 2.99 GHz, Pentium IV Hyper Threading (HT) processor, with WINDOWS XP Professional (Service Pack 2) and 512 megabyte of memory. All video footages were converted into AVI format using Adobe Premier Pro 2.0 version. Backlight was used to create maximum contrast between the insect and the substrate. Parasitoids that walked 7 cm up into one of the arms were considered to have made choice. Parasitoids not making a choice within five minutes were considered not responding. Ten adults were assessed per treatment with four replications per treatment. After testing ten individuals the tubes were cleaned and treatments were reversed to avoid position effects. The data was subjected to G test.

Electrophysiology

Electroantennogram (EAG) responses of *B. brevicornis* adults were made using a commercially available electroantennographic system (Syntech, Hilversum, The Netherlands) consisting of a dual electrode probe for antenna fixation, a CS-55 stimulus controller and an IDAC 232 box for data acquisition. The antenna was excised at scape level and then fixed with the tip to one of the electrodes and terminal portion of the club of the antennae was fixed to the other electrode as suggested by Reinecke *et al*. (2005). The antenna was fixed between the two electrodes using Spectra 360 conductive gel (Parker, Orange, New Jersey). The antenna was flushed continuously with stream of activated charcoal filtered air.

Olfactory stimulation system

The stimulus to be tested was placed into the microtip pipettes (Tarsons 100–1000 µl). This was connected to stimulus controller by silicone rubber tube. The stimulus was puffed on to the antenna by injecting the vapor phase of the micro tip pipette 15 mm upstream from the antennae in the continuous air stream (pulse time 0.5 s, continuous flow 25 ml/s, pulse flow 21 ml/s). The minimum delay between the stimulus puff was 120 s. Antennal response to host products was recorded from ten adults with three replications per antenna.

Pesticide exposure

The adult females were exposed to dry residue of insecticides. The insecticides *viz*. malathion, imidacloprid, carbaryl, that are commonly used in coconut pest management (Rajan *et al*., 2009) were obtained from Accustandards, USA. It was dissolved in acetone (Merck HPLC grade) and applied on the inner surface of the glass tubes. To obtain a homogenous residue layer 200 µl of the analyte was applied per tube. This volume aids to achieve full coverage of the internal surface of the glass tube. The tubes after filling with the insecticides were uniformly rotated over a flat surface so as to achieve a full coverage of the internal surface of the glass tube. They were left for 30 min for complete evaporation of acetone before introducing the parasitoid. Ten adults (3- 4 day old) per tube were exposed to insecticides. Three replicates were maintained per treatment. Two to three drops of honey were placed in each tube over a strip of wax coated paper. The neck of the tubes was secured by a muslin cloth so as to enable air circulation. The entire setup was maintained at room temperature 25^0C and 12: 12 h light and dark period. After 12 hrs the number of dead parasitoids was counted. For determining the regression line of mortality, four increasing doses of insecticides were used and 30 adults were exposed to each dose. The LD_{20} was estimated from the regression line and this dose was used to assess the sub lethal effect of insecticides on adult *B. brevicornis.*

Residence time on kairomone patch

The sub lethal effect of insecticides on adult female parasitoids towards host frass was determined. For this, the females exposed to pesticides at LD_{20} level were used. Those that were not exposed to pesticides served as control. Two pieces of 2 cm dia filter paper discs were placed in Petri dish. Ten mg frass (equivalent to the amount produced by one larva per day) was placed in one disc and the other was maintained as control. Individual adults were released into the Petri dish and their orientation towards the frass and the time spent on the patch was recorded using JVC TK C 1380 CCD camera, which in turn was connected to Pinnacle video capturing card in the CPU. The data was captured in 640×480 resolution, 30 frames per second and

stored in MPEG format. The time spent on the patches was calculated.

RESULTS AND DISCUSSION

Olfactory response of *B. brevicornis*

In olfactory assay the starved adults of *B. brevicornis* when provided a choice between food and host volatiles they oriented to food source (honey) (Fig. 1). In case of fed parasitoids the orientation was more to *Opisina* larval haemolymph and frass than towards food source (honey). When choice was offered between larvae and damaged leaf containing larval frass the parasitoid preferred the latter. Feeding of the parasitoids in laboratory prior to field release would aid them to search for host than wasting its energy towards foraging for food (nectar) (Fig.2). Orientation of an animal is affected by its internal condition. Host searching behavior of *Pterostichus* spp. was affected by its hunger (Wallen and Ekbom, 1994). The orientation of fed *B. brevicornis* was more towards the larval frass and haemolymph. Hexane wash of larval body and frass of *O. arenosella* elicited positive response from *G. nephantidis* and *Elasmus nephantidis* in terms of number of parasitoids entering the arm of 'Y'tube containing kairomone (Bakthavatsalam *et al*., 1999). In case of *O. arenosella* larvae that live near their feeding site, accumulation of frass can be disadvantageous as they expose themselves to natural enemies. The damaged leaf with the larval frass is used by the parasitoids as a cue to locate its host (Reddy *et al*, 2002; Rogers and Potter, 2002). Attraction of parasitoid to host frass has been observed in *Microplitis* and *Trichogramma* (Gross *et al*., 1975). Ghosh and Abdurahiman (1996) observed that gallery washings of *O. arenosella* served as a vital cue in host searching and oviposition behavior in *Apanteles taragamae.*

Antennal response of *B. brevicornis*

The antennal response of *B. brevicornis* to volatiles was assessed by electroantennography. The antennal response of females was higher than that of males. In case of female the antennal response was more for damaged leaf with frass (0.7 mv) whilst in male it was higher for the larval haemolymph (0.33 mv). Antennae of both sexes caused good responses to *Opisina* larval haemolymph followed by the volatiles form larval frass. Volatiles from undamaged leaf caused minimum antennal response in both male and female parasitoids (Table 1). The investigation on host finding by parasitic Hymenoptera is mostly done at behavioral level. The EAG experiments represent an alternative and convenient method to assess the overall sensitivity of insects to a range of compounds at physiologically relevant concentration under the rationale that the peripheral olfactory system has evolved sensitivity to behaviorally important odors (Their and Marion-Poll, 1998).

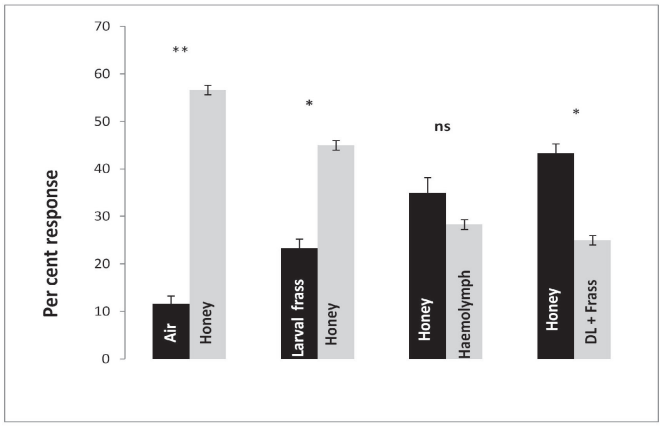

Fig. 1: Response of starved female parasitoid to odor from food source (Honey). Asterisks indicate significant difference (*p <0.05, **p< 0.01) by G test. DL – Damaged leaf Black bar represents arm-1 and grey bar represents arm- 2

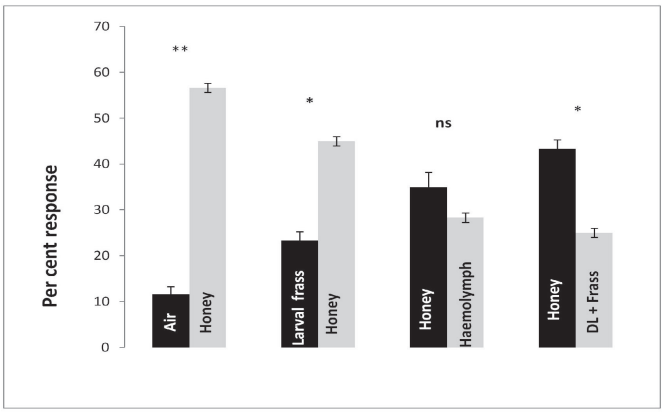

Fig. 2: Response of female parasitoid (fed) to odor from food source and host volatiles. Asterisks indicate significant difference (* p <0.05, ** p< 0.01) by G test. Black bar represents arm-1 and grey bar represents arm- 2

Table 1: EAG response of female *B. brevicornis* antennae to host associated volatiles

Stimulus	*B. brevicornis* antennal response (mV)	
	Female	Male
O.arenosella larvae	0.39 + 0.004 [d]	0.12 + 0.004 [c]
Larval haemolymph	0.67 + 0.007 [b]	0.33 + 0.009 [a]
Larval frass	0.53 + 0.008 [c]	0.28 + 0.005 [b]
Damaged coconut leaf let + frass	0.7 + 0.009 [a]	0.35 + 0.006 [a]
Undamaged coconut leaf let	0.15 + 0.008 [f]	0.08 + 0.005 [d]
Mechanically damaged coconut leaf let	0.27 + 0.007 [e]	0.15 + 0.009 [c]

Means followed by same alphabet are not significantly different by DMRT.

EAG responses do not necessarily reflect the central integrations of peripheral stimuli and therefore do not indicate the resulting behavioral responses (Baehrecke *et al*., 1989). However, there are reports of correlation of electrophysiological data with behavioral studies with minor differences (Baehrecke *et al*., 1989; Li and Dickens, 1992; Vaughn *et al*., 1996). In the present investigation, the electrophysiological antennal response of male and female *B. brevicornis* to host volatiles as well as to the volatile blend from the leaf material mechanically damaged/ herbivore-damaged leaf, showed a higher olfactory receptivity of females as compared to males. In contrast to our findings the similarity in electrophysiological responses between sexes of parasitic Hymenoptera has been reported (Lecomte and Pouzai, 1985; Salom *et al*., 1992; Li and Dickens, 1992), which suggests that several of the compounds may be used together by both sexes in host habitat/or host community locations.

Table 2: Dose mortality relationship LD values

Chemical	LD_{20}	LD_{50}
Malathion	0.65	1.81
Imidacloprid	0.45	0.91
Carbaryl	0.54	1.15

LD- lethal dose

The insecticides evaluated to fix LD_{50} values had linear regression of the dose mortality relationship. The ranking of insecticides in the order of increasing toxicity to parasitoid was imidacloprid> carbaryl > malathion. The sub lethal dose (LD_{20}) of malathion, imidacloprid and carbaryl were 0.65, 0.45 and 0.54 ppm respectively (Table 2). Parasitoids when exposed to sub lethal dose of insecticides had altered orientation to the host volatiles. The parasitoids from control group had higher response to damaged leaflet and frass volatiles as compared to fresh air (G = 30.53, 1 d.f., P=< 0.000). There was no significant difference in odor choice (air vs damaged leaflets + frass) in the parasitoids exposed to imidacloprid (0.45 ppm) and carbaryl (0.54 ppm) (G = 0.11, 1 d.f., P= 0.75; G=0.09, 1 d.f., P=0.75 respectively). In either cases < 50 % parasitoids responded to the test while majority of the parasitoids remained still without moving (Table 3). The detection of chemical stimuli and its association to adapted behavior is entirely dependent on nervous transmissions. Toxic effects of many insecticides cause perturbation of nervous transmissions, and this cause interference with kairomone perception, depending on the type of insecticide and the level of exposition (Komez *et al*., 2001).

Table 3: Effect of sublethal dose of insecticide on *B. brevicornis* orientation

Insecticide (Sublethal dose)	Per cent response of female *B. brevicornis* to arms containing		G test
	Air	Damaged coconut leaf let + Frass	
Carbaryl 0.54 ppm	14.99	13.33	ns
Imidacloprid 0.45 ppm	13.31	11.66	ns
Malathion 0.65 ppm	18.33	23.33	*
Control	13.31	58.33	**

Sublethal dose behavior relationship on patch residence time of parasitoids

The LD_{20} values of insecticides were considered as sub lethal dose for assaying the behavior of adults. Parasitoid exposed to sub lethal dose of insecticides when evaluated for their residence time in kairamone patch revealed that the control group had a residence time of over 150 sec. on the larval frass patch followed by malathion at 50 sec. Parasitoids exposed to imidacloprid and carbaryl did not spend over 10 sec in the frass patch. In majority of the cases they were immobile (Fig.3).

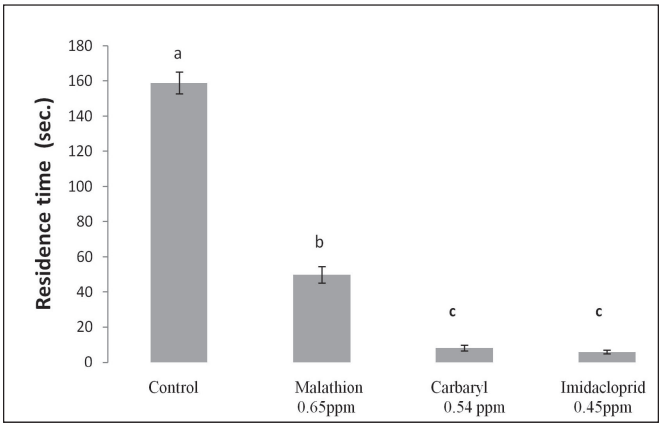

Fig. 3: Effect of sub lethal dose on patch residence by *B. brevicornis*. Means followed by same alphabet are not significantly different by DMRT.

Studies suggest that highly variable and potentially strong sub lethal effects are the general feature of the impact of synthetic organic pesticide on parasitoids. The behavior of the parasitoid exposed to insecticide was modified, but the arrestment by kairomones was still effective as seen in case of parasitoids exposed to sub lethal dose of malathion (Table 3). The modified behavior of the females exposed especially to insecticides can be explained by the mode of action of the insecticide. Chlorpyrifos interferes with the behavioral response of *L. boulardi* to kairomone of its host (Komez *et al*., 2001)

The study on the feeding status on olfactory response of *B. brevicornis* to host volatiles clearly establishes that the parasitoids have to be fed on emergence prior to field release as this would enhance their searching ability. Though sublethal dose of insecticides of imidacloprid, malathion and carbaryl doesn't cause mortality, they cause disturbance in peripheral sensory physiology of *B. brevicornis* which alters their olfactory response to host volatiles. Information gained on sub lethal dose will help to time the safe interval for release of *B. brevicornis* in pesticide treated fields.

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Zinc nitrate derived nano ZnO: Fungicide for disease management of horticultural crops

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ABSTRACT

A laboratory study was undertaken to assess the antifungal property of the nano ZnO against a food borne pathogen *Aspergillus fumigatus*. In this study nano ZnO was synthesized from zinc nitrate solution using sodium hydroxide. Synthesized nano ZnO was characterized by DLS, SEM, TEM and EDAX analysis. Nano ZnO was used for antifungal assay against *A. fumigatus* through poisoned food technique. Antifungal activity of nano ZnO was found to be significantly higher than bulk sized ZnO. Mechanism of action studies of nano-ZnO on the *A. fumigatus* show that hydroxyl radicals and superoxides generated by nano-ZnO remain bound to the fungal cell wall and causes deformity due to high energy transfer leading to death of fungi.

Key words: Nano ZnO, *Aspergillus fumigatus*, fungicide, agriculture, horticulture

INTRODUCTION

Different agro-climatic regions of India is highly supportive of a large number of horticultural crops (Roy and Thorat, 2008; NHB, 2005 and Wahl, *et al.*, 2006).

Though India is the second largest producer of horticultural crops, next to China, India has always performed poorly in exporting its produce to international markets. One of the major reasons for the failure is inability to meet the international biosafety standards. One of the major pathogen affecting horticultural produce is endophytic fungus, *Aspergillus fumigatus*. This fungus affects nearly all fruits and vegetable crops and stays alive in the final produce form. *A. fumigatus* is the major cause of Aspergillosis in immuno-compromised patients. *A. fumigatus* contaminated horticultural produce are not accepted by foreign markets due to potential health risk of the customers. Therefore, control of this fungus will be major boost for Indian horticulture. Nanotechnology offers a major promise towards control of *A. fumigatus* (Hunag, W.S. 2004, Economic Survey 2006-07, Lee, Y.S. *et al*., 2008) .

Nano metal oxides are used in different areas like solar energy conversion (Leschkies, K.S. *et al.*, 2007, Breeivik, T.H. *et al.*, 2007, Dhoke, S.K. *et al.*, 2009), super conductors (Dong, L.F. *et al*., 1997), chemical sensors (Liang S.W. *et al*., 2009) magnetic devices (Li, J.F. *et al*., 2001, Jung, S.W. *et al*., 2003, Li, S.C. and Li. Y.N. 2010), due to their promising activities in the corresponding areas. Nano ZnO is one of the five zinc compounds that are currently listed as, generally recognized as safe (GRAS) by US food and Drug Administration (21 CFR 182.8991). Previously nano ZnO was used as antibacterial agent (Ma,X.Y. and Zhang, W.D., 2009 and Latge J.P., 1999) against *E. coli, S. oneidensis, S. aureus* etc, but we used nano ZnO as a fungicide first time against *A. fumigatus.* Inhalation of conidia by immunocompromised patients has a number of major adverse effects, since the conidia are not eliminated relatively efficiently by innate immune mechanisms. Thus, *A. fumigatus* is viewed as a potent pathogen responsible for allergic forms of the disease, such as farmers' lung, a clinical condition observed among individuals exposed repeatedly to conidia, or aspergilloma, an overgrowth of the fungus on the surface of pre-existing cavities in the lungs of patients treated successfully for tuberculosis. Aspergilloma, commonly referred to as "fungus ball," occurs in pre-existing pulmonary cavities that were caused by tuberculosis, sarcoidosis, or other bullous lung disorders and in chronically obstructed paranasal sinuses (Wu, C. *et al*., 2006) Conidia are also involved in mild aspergillosis with symptoms like cough, coughing up blood or brownish mucus plugs, fever, wheezing and severe weight loss. Patients with acute aspergillosis, show symptoms like blood in the urine, bone pain, chest pain, chills, decreased urine output, severe headache, increased phlegm production (which may be bloody), shortness of breath, skin sores (lesions) and also develop vision problems. In this paper we demonstrate that

biosafe form of nano ZnO derived from zinc nitrate act as an effective antifungal agent against *A. fumigatus*.

MATERIALS AND METHODS

Chemicals

AR grade zinc nitrate [$Zn(NO_3)_2 \cdot 6H_2O$], Sodium hydroxide (NaOH) were purchased from Merck, India. AR grade Potato Dextrose Agar (PDA) was purchased from Himedia, India. Deionised water (Sartorious Stedim biotech) was used throughout the experiment.

Fungus Strain

Wild *Aspergillus fumigatus* strain was purchased from Microbial Type Culture Collection (MTCC), Chandigarh, India. Strain was phenotypically and genotypically characterized in the laboratory with standard markers and PCR primers.

Synthesis of nano ZnO nanoparticles

To prepare the precursor, 50 ml of 0.5 M zinc nitrate was added (drop wise) to 50 ml of 0.5 M NaOH solution within a time period of 30 min, and then the mixed solution was kept at 25 °C for 2 h under vigorous stirring condition. The aqueous solution turns slimy, and precipitates were found in the bottom of the flask. Finally, the white precursor was collected after the mixture was centrifuged, filtrated, washed with water and alcohol, and dried at room temperature under vacuum overnight for further characterization (Dhingra and Sinclair, 1985).

Characterization of ZnO nanoparticles

Synthesized nano ZnO was characterized by using Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and EDAX analysis. Particle size distributions (PSD) of synthesized nano ZnO was recorded from Dynamic Light Scattering (DLS) in a particle size analyzer (90 Plus, Brookhaven Instrument Corporation, USA). The laser light (λ = 660 nm) was scattered at an angle θ = 90º at 25 ºC placing the dispersion in a glass cuvette. For this measurement a small portion of sample solution in ethanol was taken in a test tube and then diluted with further addition of ethanol and then again sonicated for 30 minutes in a sonication bath (Branson Inc, USA). After settling for 10 minutes the solution was again diluted with same ratio of ethanol and measurements were performed after sonication of the solution for 30 minutes. The average size was found to be 586 nm (~ 600 nm) with poly-dispersity ratio of 0.242 (Fig. 1a). Scanning electron microscope was used to study the size and surface morphology of ZnO nanoparticles. Due to small size of the particles the morphology was not quite clear from SEM micrograph (Fig. 1b). However, the size of the particles was far smaller (~50 nm) as compared to the DLS results. Composition of the sample was confirmed by Energy-dispersive X-ray spectroscopy. EDAX analysis showed that the sample contained Zn and O as the elemental composition of synthesized nano ZnO (Fig. 2a). Distinct size and shape (50 nm) was confirmed by TEM micrograph (Fig. 2b). Taken together the size of nano-ZnO was found to be 50 nm.

Antifungal assay

Wild strain of *A. fumigatus* was used for antifungal assay. Strain was maintained on potato dextrose agar plates at 30°C by periodical sub-culturing. The antimycotic activity of both nano ZnO and micronized (bulk) were tested at four different concentrations (250, 500, 1000 and 2000 ppm) using modified poison food technique originally proposed by Dhingra and Sinclair in 1985 (Stoimenov, P.K. *et al*., 2002). Every concentration of micronized and nano ZnO

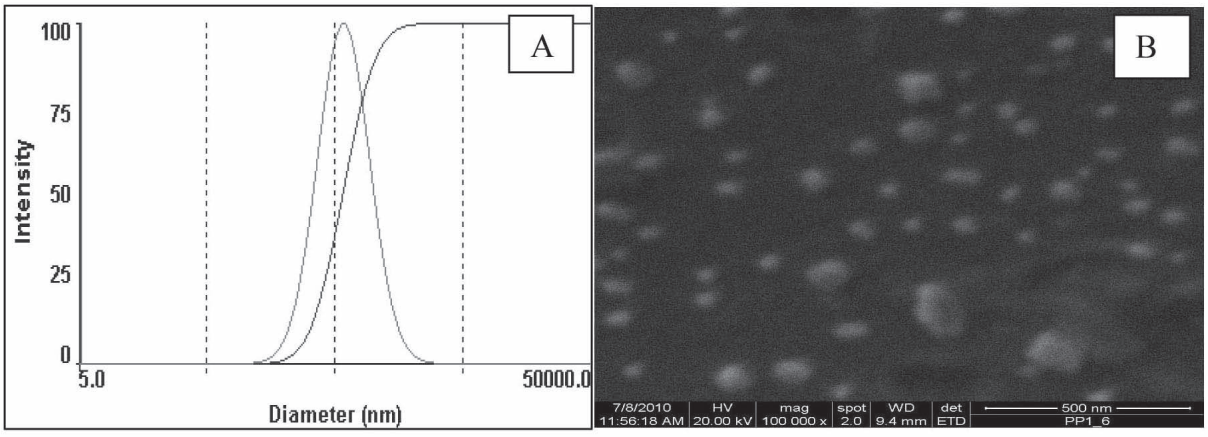

Fig. 1a: Particle size distribution (DLS measurements) of nano ZnO which depicts the size distribution as well as the quality of the sample; **Fig. 1b:** Scanning electron microscopic image of nano ZnO.

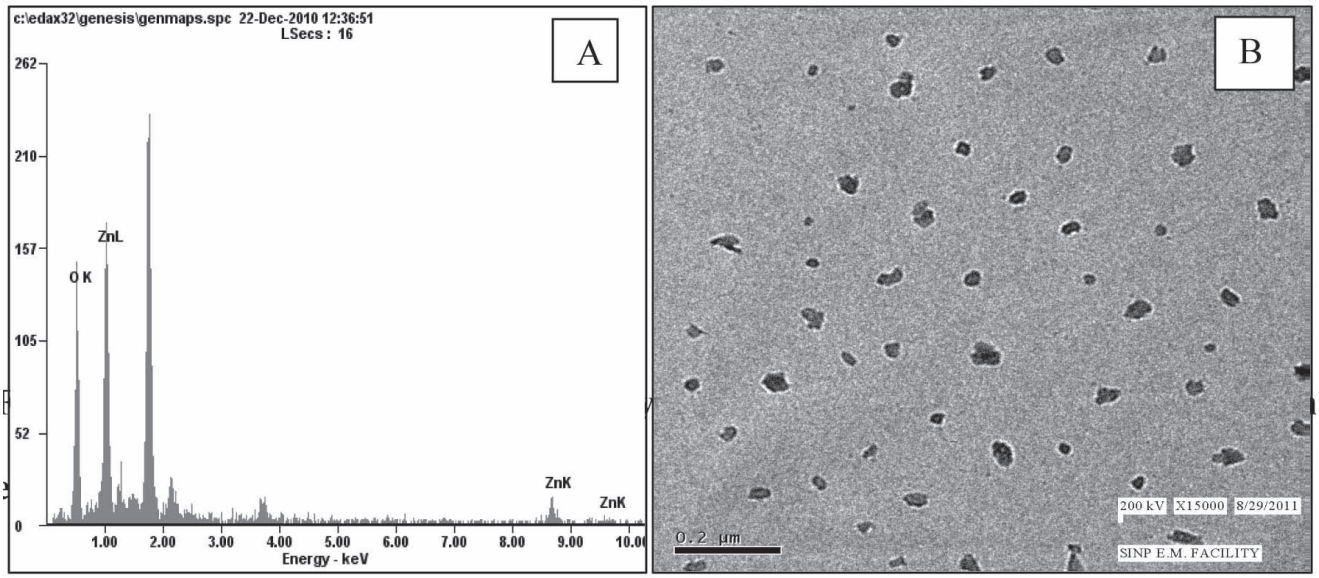

Fig. 2a: Energy-dispersive X-ray analysis of nano ZnO;

Fig. 2b: Transmission electron micrograph of nano ZnO.

was mixed in distilled water and sonicated for 15 minutes and then mixed with potato dextrose agar. Fungal suspension (3µl) was spotted in triplicate per Petri plate and incubated at 28°- 2 °C for 48hrs. Radial growth of the fungus was measured manually by zone diameter measuring scale. Change in zone diameter between control and treatment plates with increasing concentrations of micronized and nano ZnO was analyzed statistically.

Scanning electron microscopic study *A. fumigatus:* Glass slides required for microscopic study were prepared following standard method (Yajoi, H. 2005). For SEM study, fungal culture was prepared by standard protocol (Padmavathy, N and Vijayaraghavan, R., 2008). Briefly fungal cultures (both control and nano ZnO treated) were fixed with 2% glutaraldehyde solution at 4 °C for 2 hours. After that, specimens were post fixed for 2 hours with 1% osmium tetroxide solution. Then samples were dehydrated with graded ethanol (Fang, M. *et al.*, 2006)

RESULTS AND DISCUSSION

The fungicidal efficacies of ZnO suspensions (both nano and bulk) at four different concentrations (250 ppm, 500

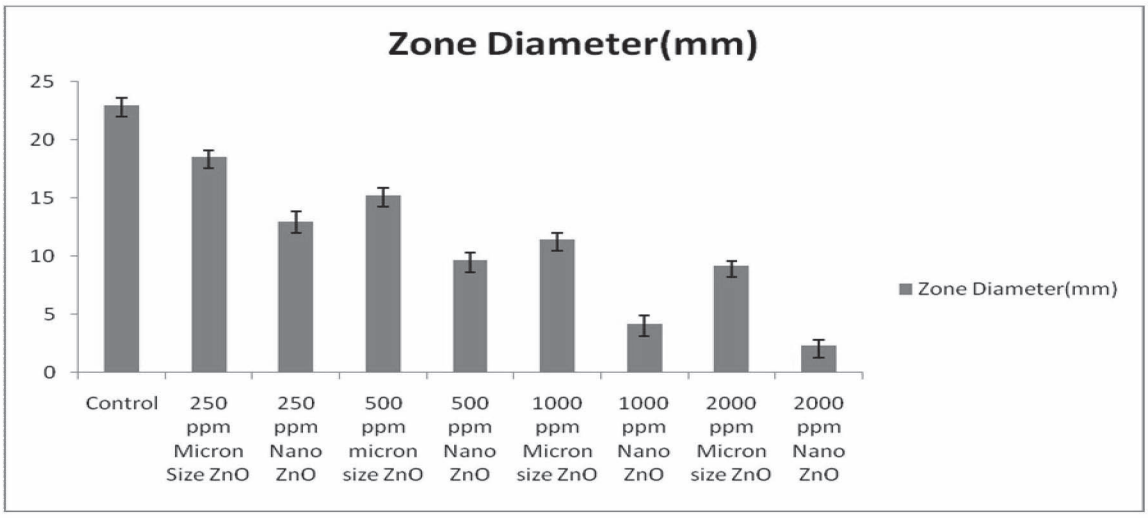

Fig. 3: Reduction in radial growth (means of zone diameter ± standard deviation) among *A. fumigatus*, after treatment with control (without any particles), micron size ZnO and nano ZnO.

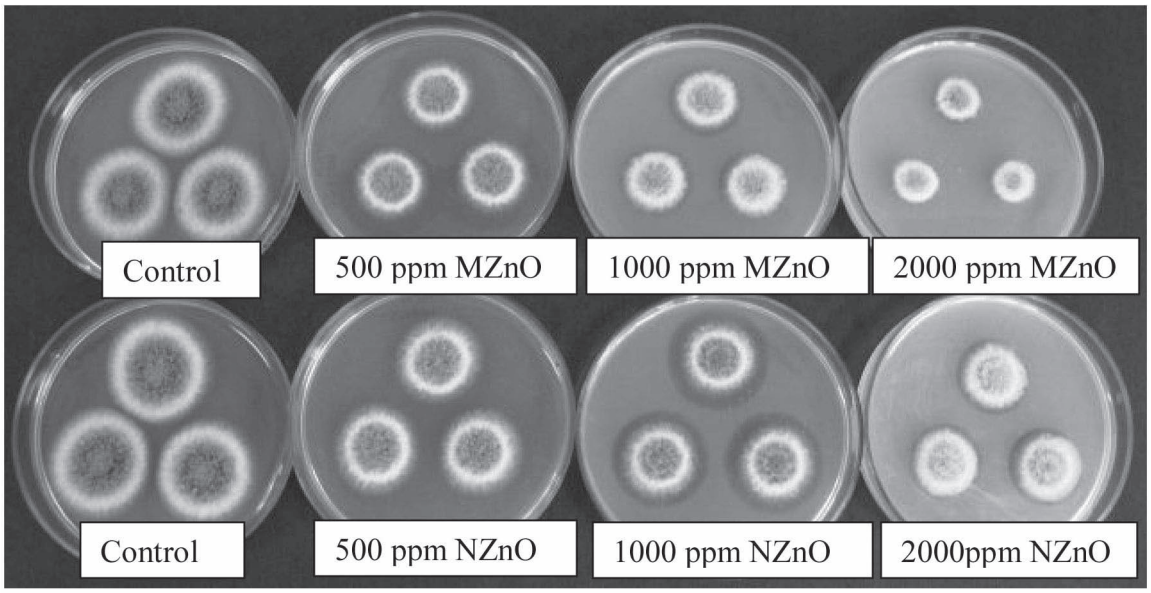

Fig. 6: Photographs of comparative study of zone diameter of *A. fumigatus* treated with Micron size ZnO (MZnO) and Nano ZnO (NZnO) after 48 hours of inoculation.

ppm, 1000 ppm and 2000 ppm) after 48 h of inoculation are shown in Fig. 3 and 4.

Table 1: Statistical representation of reduction of radial growth of A. fumigatus after treatment with water (Control), Micron size ZnO (MZnO) & Nano-ZnO (ZNPs) at different concentrations

Treatment	Zone Diameter(mm) ±	SE
Control	22.96 ±	0.62
250 ppm Micron Size ZnO	18.55 ±	0.50
250 ppm Nano ZnO	12.96 ±	0.90
500 ppm micron size ZnO	15.20 ±	0.67
500 ppm Nano ZnO	9.62 ±	0.67
1000 ppm Micron size ZnO	11.4 ±	0.56
1000 ppm Nano ZnO	4.10 ±	0.77
2000 ppm Micron size ZnO	9.17 ±	0.38
2000 ppm Nano ZnO	2.27 ±	0.52

The data (Table 1) showed that nano ZnO is more effective than its micron counterpart. This can be explained on the basis of the oxygen species released on the surface of ZnO, which cause fatal damage to microorganisms. The generation of highly reactive species such as OH^-, H_2O_2, and O_2^{2-} is explained as follows. Since ZnO with defects can be activated by both UV and visible light, electron–hole (e^-h^+) can be created. The holes split H_2O molecules (from the suspension of ZnO) into OH^- and H^+. Dissolved oxygen molecules are transformed to superoxide radical anions ($\cdot O_2^-$), which in turn react with H^+ to generate ($HO_2^\cdot$) radicals, which upon subsequent collision with electrons produce hydrogen peroxide anions. The generated H_2O_2 can penetrate the cell membrane and kill the fungus.

$ZnO + h\upsilon u \rightarrow e^- + h^+$; $h^+ + H_2O \rightarrow OH + H^+$

$e^- + O_2 \rightarrow O_2^-$; $\cdot O_2 + H^+ \rightarrow HO_2^\cdot$

$HO_2^\cdot + H^+ + e^- \rightarrow H_2O_2$

Since, the hydroxyl radicals and superoxides are negatively charged particles, they cannot penetrate into the cell membrane and must remain in direct contact with the outer surface of the fungus, however H_2O_2 can penetrate into the cell. The surface area to volume ratio of nano ZnO is higher than bulk ZnO. The generation of H_2O_2 depends strongly on the surface area of ZnO, which results in more reactive oxygen species on the surface and thus the higher antimicrobial activity of smaller nanoparticles. ZnO suspensions in the lower concentration range seem to exhibit less antimicrobial activity which may be due to the possible presence of fewer Zn^{2+} ions, which might act as a nutrient.

In SEM study, severe damage to cell wall was observed in case of nano ZnO treated fungal hyphae (Fig. 5A) in comparison to control hyphae of *A. fumigatus* (Fig. 5a). Healthy, regular, uninterrupted cell surface of control hyphae was observed while nano ZnO treated hyphae showed surface depression of cell wall. A dramatic change of hyphal cell surface was observed in nano ZnO treated hyphae. As discussed above, nano-ZnO produces hydroxyl radicals and superoxides which are negatively charged particles and therefore cannot penetrate into the cell membrane. As a result, they remain in direct contact with the outer surface of the fungus. These free radicals cause deformity in the outer membrane of the fungi. Morphological changes such as disappearance of smooth surface texture and thickening of cell wall confirms that

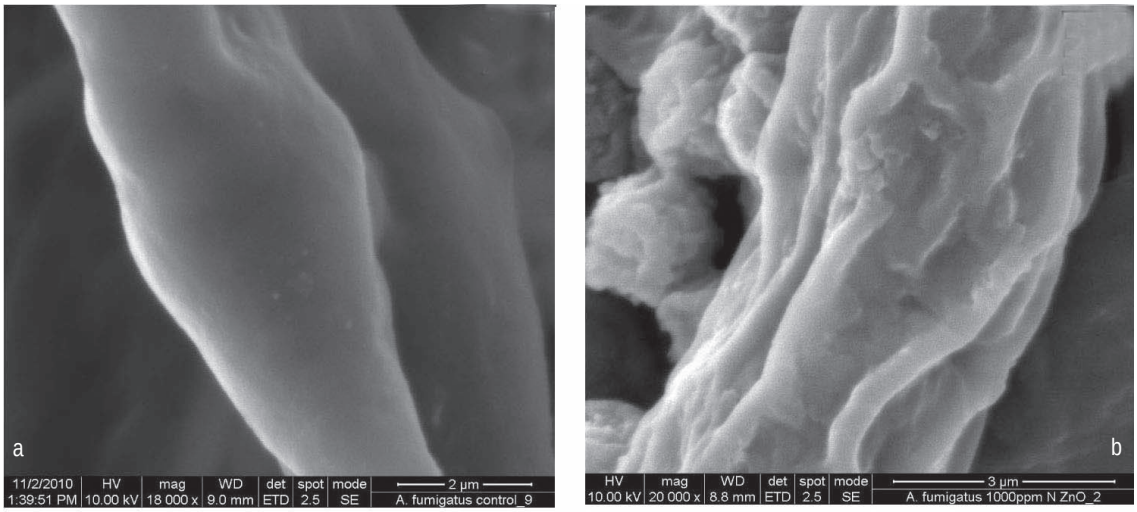

Fig. 5: Scanning Electron Micrographs of control (a) and nano-ZnO treated hyphae (b) of *A. fumigatus.*

nano ZnO possibly act on the cell wall of fungal hyphae (Fig. 5b).

The study suggests that nano ZnO showed a greater inhibition than bulk (micron sized) against food borne pathogen *A. fumigatus*. We hypothesized that the cell wall rupture must be due to surface activity of ZnO in contact with fungus. The damage of the cell wall and cell membrane leads to the leakage of minerals, proteins and genetic material causing cell death. Therefore, Zinc nitrate derived nano ZnO acts as biologically safe fungicide against *A. fumigatus*. The nano-formulation utilized during the study can be sprayed on the crop surface and thus can protect the plant from *A. fumigates* infection. This novel fungicide if commercialized would be a major boost for horticultural crop production in India.

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Analysis of growth related traits in turf grasses in India

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ABSTRACT

The present investigation conducted on eight species and four varieties of turfgrasses depicted significant differences in all the growth related traits studied. All turfgrasses under study exhibited fine leaf texture except *Eragrostis curvula* (Schrad.) Nees, having medium coarse texture, *Paspalum notatum* Flugge and *Argentine bahia* having coarse texture and *Poa pratensis* L. which exhibited medium fine leaf texture. The *Agrostis palustris* L. exhibited maximum mean performance for shoot density/10 cm^2 (277.33) whereas minimum was exhibited by *Cynodon dactylon* L. var Panama (57.67). Maximum root density/10cm^2 was observed in *C. dactylon* L. var. Panama (15.80) followed by *C. dactylon* L. var. Panam (14.87).

Key words: Turfgrass, qualitative and quantitative traits, shoot density, species.

Turf grasses are considered as an integral part of landscape ecological systems worldwide which provide aesthetic value (Roberts *et al.*, 1992). Turf grasses are narrow-leaved grass species that form a uniform and long-lived ground cover which can tolerate traffic and low mowing heights (Rongda *et al.* 2008). Turf grass is world widely used in enhancing and maintaining the function and beauty of lawns, aesthetic fields, etc. (Fender, 2006; King and Balogh, 2006), where proper selection and care of turf grass depends upon knowledge of the environmental adaptations, cultural requirements and quality features of the grass species. Most of the work on turf grasses has been done in foreign countries viz., USA, Australia, Japan, Singapore, etc., but these grass species and varieties have not proved suitable for Indian agro-climatic conditions because a variety bred under a specific climatic zone, may not necessarily perform well under other climatic zones. Some preliminary work has been carried out concerning this but less information is available on these aspects in India. In order to contribute towards improvement in turf grasses, the objective of this study was to evaluate turfgrass species and varieties for various growth related qualitative and quantitative traits.

The plant materials utilized for the present study consisted of eight species and four varieties of turf grasses named as *Agrostis palustris* L., *Eragrostis curvula* (Schrad.) Nees, *Dichondra repens* Forst, *Paspalum notatum* Flugge, *Argentine bahia*, *Poa pratensis* L., *Cyndon dactylon* L. var bargusto, *C. dactylon* L. var. Palna, *C. dactylon* L. var. Panam, *C. dactylon* L. var. Panama, C. *dactylon* L. var. Selection1, *Lolium perenne* L. The seeds of turfgrass species were procured from NBPGR, New Delhi. The present investigation was carried out at Research Farm of Division of Floriculture and Landscaping, Indian Agricultural Research Institute, New Delhi after rainy season 2011 in a randomized block design with three replications. The above farm is situated at 77°12'E longitude 28°40'N latitude and at an altitude of 228.16 m above the mean sea level. The observations were recorded on various turf grass quantitative traits viz., germination percentage (%), shoot Length (cm), root length (cm), fresh weight of shoots (g), fresh weight of roots (g), dry weight of shoots (g), dry weight of roots (g), shoot density per 10 cm^2, root density per 10 cm^2, dry root/shoot ratio, relative water content (RWC – %).

All turf grasses under study exhibited fine leaf texture except *Eragrostis curvula* (Schrad.) Nees having medium coarse texture, *Paspalum notatum* Flugge and *Argentine bahia* having coarse texture and *Poa pratensis* L. which exhibited medium fine leaf texture. Most of the turf grasses exhibited spreading growth habit except *Eragrostis curvula* (Schrad.) Nees and *Lolium perenne* L. having upright growth habit. Mean performance of the different species revealed that no single species was superior for all traits (Table 1). The data revealed a high range in shoot length (2.06-15.4 cm), root length (1.20-8.67 cm), relative water content (54.35-86.41), shoot density per 10cm^2 (57.67-277.33) and root density per 10cm^2 (2.83-15.80). The longest shoot was recorded in *Eragrostis curvula* (Schrad.) Nees (15.4 cm) followed by *Lolium perenne* L. (13.66 cm) and *Cyndon dactylon* L. var Bargusto (12.4 cm) whereas *Argentine bahia* exhibited shortest shoots (3.1 cm). This might be due to various environmental and genetic factors which influence the shoot growth rate, one of them being ethylene production which has been reported to influence

Table 1: Mean performance of turf grass species for growth related traits

Species	Shoot Length (cm)	Root length (cm)	Fresh weight of shoots (g)	Fresh weight of roots (g)	Relative water content (%)	Shoot density /10cm ^{2}	Root density /10cm ^{2}
Agrostis palustris L.	7.73	3.13	0.04	0.023	56.69	277.33	3.63
Eragrostis curvula (Schrad.) Nees	15.40	8.67	0.11	0.028	77.02	108.00	2.83
Dichondra repens Forst	3.66	2.60	0.25	0.020	82.56	236.67	7.33
Paspalum notatum Flugge	2.06	1.30	0.09	0.017	65.20	128.00	8.73
Argentine bahia	3.10	1.43	0.08	0.018	84.14	114.00	11.33
Poa pratensis L.	7.10	4.60	0.06	0.024	70.35	127.33	7.13
Cyndon dactylon L. var bargusto	12.40	6.60	0.15	0.013	77.73	102.67	9.97
C. dactylon L. var. Palma	5.30	1.40	0.13	0.082	68.49	215.00	11.60
C. dactylon L. var. Panam	10.70	5.63	0.08	0.031	71.07	71.00	14.87
C. dactylon L. var. Panama	11.60	8.00	0.10	0.039	72.24	57.67	15.80
C. dactylon L. var. Selection1	5.70	1.20	0.05	0.016	54.35	110.33	5.90
Lolium perenne L.	13.66	4.73	0.16	0.035	86.41	207.67	9.47
Mean	8.20	4.11	0.11	0.037	72.19	146.30	9.05

the shoot growth in turfgrasses under varied stress conditions (Verslues *et al.*, 1996). Root length is considered to be important survival factor of turfgrass growing in areas of limited water (Simanton and Jordan, 1986). Therefore, during the experiment, observations on root length and dry root : shoot ratios were also recorded. *Eragrostis curvula* (Schrad.) Nees showed maximum root length (8.67 cm) followed by *Cynadon dactylon* L. var. Bargusto (6.6 cm). However, minimum root length was observed in *C. dactylon* L. var Selection 1 (1.2 cm) which was at par with *Paspalum notatum* Flugge (1.3 cm), *C. dactylon* L. var Palma (1.4 cm) and *Argentine bahia* (1.43 cm). Rooting characteristics of turfgrasses have a profound influence on their response to abiotic stresses and this has been supported by many earlier reports. The turf grasses having more root length, require less water i.e. more tolerant to drought. Rooting depth is more important than total root production in selection for drought avoidance (Erusha, 1986; Sullivan, 1983; Garrot and Mancino, 1994; Carrow 1996). White *et al.* (1993) observed that based on root length characteristics, drought resistance vary from one turf grass specie to other.

Since, shoot density is a key trait to assess the turf quality, therefore, shoot density was measured during evaluation of various turf grasses. The highest shoot density per 10cm^2 was exhibited by *Agrostis palustris* L. (277.33) which was at par with *Dichondra repens* Forst (236.67) while lowest was found in *C. dactylon* L. var. Panama (57.67) which was at par with *C. dactylon* L. var. Panam. Maximum root density per 10 cm^2 was observed in *C. dactylon* L. var. Panama (15.80) followed by *C. dactylon* L. var. Panam (14.87) which were at par to each other. However, minimum was exhibited by *Eragrostis curvula* (Schrad.) Nees (2.83) which was at par with *Agrostis palustris* L. (3.63).

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NRCSS Ani-1 — A new variety of Anise

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ABSTRACT

Anise (*Pimpinella anisum* L.) well-known for several functional properties, is valued abundantly in food, medicinal and perfumery industry. To cater the needs of growers, processors and exporters, the first variety of anise NRCSS Ani-1, has been developed. This variety is high yielding (average yield, 733 kg/ha), bears attractive seeds with a high volatile oil content of 3.2 % and anethole content of 89.6 %. This variety offers a potential nutraceutical value and scope for diversifying the farming systems in the semi-arid agro-ecosystem, where anise forms one element.

Key words: Anise, *Pimpinella anisum*, cultivar, essential oil

INTRODUCTION

Anise (*Pimpinella anisum* L., family Apiaceae) is an annual seed spice, grown mostly for the seeds (botanically fruits). The fruits are used for flavouring food, confectionery, chewing gums, beverages, mouthwashes and toothpastes. Essential oil is the prized product of anise seeds and is used in food, medicinal and perfumery industry owing to several functional properties (Ozguven, 2001, Haghir *et al*., 2010). Anise breeding is still nascent in India and NRCSS Ani-1 is the first result of the pioneering efforts of National Research Centre on Seed Spices (NRCSS) Ajmer, Rajasthan. It has been identified for release under All India Co-ordinated Research Project on Spices. It is a high yielding variety, bearing attractive seeds with a high volatile oil content of 3.2 %. The anethole is the main constituent of essential oil and in NRCSS Ani-1, the anethole content is 89.6 %. This variety is suitable for cultivation in semi-arid region under irrigated conditions and takes 162 days to reach maturity. It gives an average yield of 733 kg/ha under semi-arid conditions with potential yields of 1150 kg/ha (Malhotra, 2006). This variety suits best in diversifying the farming systems in the semi-arid agro-ecosystem, where anise forms an important component.

ORIGIN AND DESCRIPTION

NRCSS Ani-1 (Sel A Ani-01-2) originated through selection from EC 22091 at NRCSS, Ajmer. The breeding programme aimed to create a cultivar of anise suitable for cultivation under semi arid conditions. Till the development of this variety, no other improved variety is available except for a report for superior line EC 22091 (Pareek *et al*., 1981). The plants of variety NRCSS Ani-1, grows to a height of 56.2 cm with primary branches of 6.5 in number and thus is suitable for sowing in close proximity at distance of 30 cm between rows and 15 cm between plants. The leaves are dark green in colour. The lower leaves are broadly triangular, incised serrate and the upper ones are trifid. The variety takes 103 days to reach 50% onset of flowering. On the basis of days taken to seed maturity, the anise varieties can be categorized into three groups i.e. early (125-145 days), mid (145-165 days) and late (>165 days). This variety tends to be 12 days early in maturity than the check (174 days) but falls under the category of mid group taking 162 days to attain seed maturity stage. The plant bears profuse inflorescence with around 52.6 umbels per plant and 15.2 umbelets per umbel at about 75% flowering stage whereas 18.4 fruits per umbellate were counted at full seed set stage when fruits are green. Salient ancillary observations on growth and yield attributes are given in Table 1 and Fig.1. According to the average yield performance obtained in four consecutive years from 2002-03 to 2005-06, the selection A Ani-01-2 renamed as NRCSS Ani-1 exhibited 733 kg fruit yield per ha which is 23.4 % higher than check a line recommended for cultivation in India during early eighties (Pareek *et al*., 1981) (Table 2). The variety consistently performed well to the other test varieties at all the test locations (Table 2). The fruits are attractive and bold (2.7 g of 1000 seeds) and are brown, medium in size with volatile oil content up to 3.2%. The anethole content an important valued criteria for the essential oil quality, is present to the high level (89.6%).

Table 1: Ancillary observations of Anise Varieties (Mean of 2002-03 to 2005-06)

Sl. No.	Characters	A Ani-01-1	A Ani-01-2(NRCSS Ani 1)	A Ani-01-4	EC 22091
1.	Days to 50% flowering	106	103	102	111
2.	No. of primary branches/plant	5.8	6.5	6.2	5.6
3.	No. of umbels/plant at 75% flowering stage	48.3	52.6	43.6	42.4
4.	No. of umbellates/umbelat 75% flowering stage	13.2	15.2	14.3	12.9
5.	No. of seeds/umbellate at full fruit set stage	14.8	18.4	14.2	14.8
6.	Plant height (cm)	52.4	56.2	48.6	48.8
7.	Days to maturity	168	162	162	174
8.	1000 seed weight (g)	2.2	2.7	2.5	2.1
9.	Essential Oil (%)	2.9	3.2	3.0	3.2
10.	Anethol (%) in total oil*(This estimation relates to only year 2005-06)	78.4	89.6	90.0	90.2

Table 2: Yield performance of Anise in varietal evaluation trial during four years

S.No.	Entry	Seed yield kg/ha				Average yield kg/ha	% increase over control
		2002-03	2003-04	2004-05	2005-06		
1.	A Ani-01-1	645.4	618.5	712.4	682.5	664.7	The selection A Ani-01-2 have shown 23.4 % higher yield on the check
2.	A Ani-01-2(NRCSS Ani 1)	684.2	748.5	738.4	763.3	733.6	
3.	A Ani-01-4	630.8	645.7	681.8	622.5	645.2	
4.	EC 22091Check	596.7	612.4	567.3	601.4	594.4	
	CD at 5%	63.8	84.4	92.6	88.6	79.4	

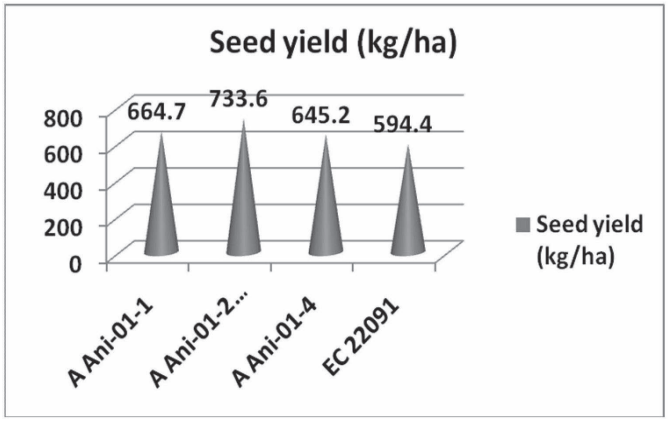

Fig. 1: Seed yield performance of anise varieties

Anise seeds are used as food flavourant (Var. NRCSS Ani 1)

A view of Anise plant (NRCSS Ani 1)

According to earlier reports, the variation in essential oil estimated for 29 samples from different geographic areas ranged between 1.3 to 3.7% and trans anethol from 78.63 to 95.21% (Arslan *et al.,* 2004) and 76.9 to 93.7 (Orav *et al.,* 2008). The considerable high quantity of essential oil and anethole, the peculiar fragrant principles, thus attributes for presence of high pleasant fragrance, tends the variety NRCSS Ani-1 more suitable for industrial and export purpose. Therefore, this variety offers a potential nutraceutical value and scope for cultivation in the semi-arid agro-ecosystem.

HOW TO GROW AND PROCESS

A fairly warm weather during sowing with a temperature of 20-28 ^{o}C is desirable. Mid October is ideal for sowing in North Indian plains. The crop is raised through seeds requiring 8 kg^{-1}ha, drilled in rows at a distance of 30 cm and keeping plant to plant spacing of 15 cm with in rows, seed depth should not exceed 2.5 cm. The seed starts germinating after 7 days and the germination end by 10 days. The crop is moderate in its manure and fertilizer requirements. The flowering and grain filling are important stages requiring irrigation. The umbels become ready for harvest in March. The terminal umbels mature first and lateral ones after 15 to 20 days gap. Average yield of 6.5-7.5 quintals per hectare can be obtained. Anise should be crushed and distilled immediately after harvest to obtain higher oil yield. The freshly harvested seeds contain more oil. The fresh seed crop generally yields 3.2% oil. Anise oil is a colourless or pale-yellow liquid having market value for the characteristic odour and taste.

AVAILABILITY

Limited quantity of breeder seed of this variety is available for testing and commercial growing at NRCSS, Ajmer. For further details, the section of Technology Commercialization of NRCSS, Tabiji, Ajmer may be contacted (nrcss.director@gmail.com).

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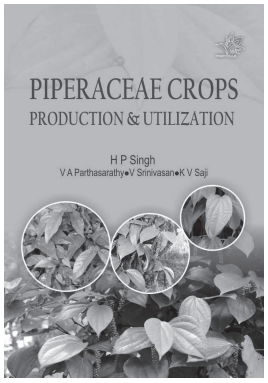

Piperaceae Crops – Production and Utilization

H.P. Singh, V.A. Parthasarathy, V. Srinivasan and K.V. Saji
2012, ISBN 978-81-85873-75-6, Rs 1,350/US$ 60
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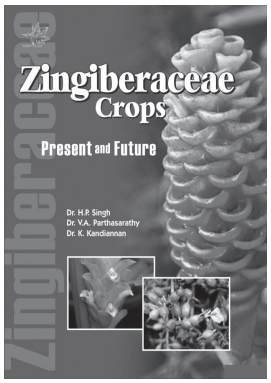
This is one of the very useful and interesting monographs on the very useful but less known family of very important crops. The family consists of 5 genera and about 3600 species. The well-known examples of useful plants of this family are Black pepper and Betel vine besides many other members. The plants have a global distribution and many have rich ethno botanical and ethno pharmaceutical history. The book has been well organised into 13 chapters written by eminent authors. A total of 26 eminent authors have contributed to various chapters. The first chapter introduces lucidly the family and the crops. The treatise on botany is excellent, covering all aspects of taxonomy and cytogenetics. Improvements of these crops using conventional and biotechnological tools have been dealt in detail. The production and agronomy have been covered in details along with nutrition and physiology. The crops dealt with are useful as spices and as medicine. The chapters on chemistry and nutraceutical uses have been dealt with adequately. A chapter on the use of these crops in traditional Ayurvedic medicine makes an interesting reading. A separate chapter covers the under exploited crops in the family.

An exhaustive attempt has been made to cover all the important aspect of the Piperaceae family. The editors deserve complement for bringing out such an important book. While books are available on many important crops, the crops dealt within the present book are hard to find. A very dedicated attempt has been made with a clear index at the end. It would be a welcome addition to all the libraries in botany, agricultural laboratories and educational institutes. All those working on spices and medicinal plants and various stakeholders will find this monograph very useful. I congratulate the editors for the fine contribution.

I have strongly believe that this book would be highly beneficial for botanist, horticulturists, agriculturists, students, growers, teacher and various stakeholders.

K.V. Peter
Former Vice Chancellor, KAU
Vellayani, Kerala



Zingiberaceae Crops – Present and Future

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The book entitled "*Zingiberaceae* Crops – Present and Future" is a monograph on the Zingiberaceae family which is one of the largest families under monocots. This family consists of about 53 genera and more than 1300 species comprising of many economically important plants like ginger, cardamom, turmeric and many ornamental and medicinal plants. The book has been very nicely structured and contains sixteen chapters. The chapters have been contributed by eminent persons in the relevant field. The first chapter introduces the theme very graphically on production trend and the importance of these crops. The various chapters on botany, cytogenetics, genetic diversity and biotechnology address the basic aspects of the crop. It would be a very valuable reference for breeders, biotechnologists and students. It provides vistas for future research as well. A chapter on physiology is well depicted. Many such monographs do not carry such a detailed account on physiology. Cropping zones using most modern tools of GIS have been described well. The agronomical aspects of these groups of crop have been dealt well. The editors have not lost sight of important area. Production techniques, input use efficiency, management of pests and diseases and have been covered precisely in four different chapters. Many of these crops are essentially used as spices and as medicinal plants. The chemistry of these crops has been dealt in detail along with the chapter on Post Harvest processing. Nutraceutical, pharmaceutical and ayurvedic uses have been dealt in two chapters. A welcome chapter, unusual in many other monographs, is on web resources.

This book has been very lucidly written with an exhaustive review clearly giving future directions. I strongly recommend this book to all students of horticulture/agriculture in general and students of spices in particular in the Agricultural/Horticultural universities. It would be of great use to the biologists, botanists, horticulturists and even to the growers. In short, this book should find place in all the agricultural/bioscience libraries.

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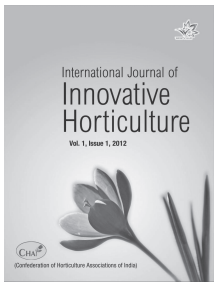

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