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CONTENTS

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Cultural and genetic evaluation of *Cochliobolus sativus* during successive passages through susceptible barley

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Key words: *Cochliobolus sativus*, *Hordeum vulgare*, phenotypes, spot blotch.

Abstract: The objective of this work was to assess the stability of retrotransposons DNA elements and several key phenotypic traits important for virulence of *Cochliobolus sativus* after serially transferring through susceptible barley plants. A significant increase in virulence was observed in offspring isolates generated from the aggressive isolate Pt4, in contrast to the lack of significant changes in those obtained from the weakly aggressive isolate Pt1 after seven successive passages. No apparent differences in phenotypes, including mycelial growth, conidiation and conidial germination were observed among isolates from the same parent isolate on artificial medium. Based on retrotransposon microsatellite amplified polymorphism (REMAP), parents and their generations were identical during the serial transfers. Taken together, our results suggest that all single-conidials of the parents and their generations were stable genotypically during seven serial transfers with a change in virulence of the aggressive isolate generations.

1. Introduction

Cochliobolus sativus (Ito & Kurib.) Drechsl. ex Dast. [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.] is an ascomycetous fungus that causes spot blotch (SB) of barley, *Hordeum vulgare* L., a disease responsible for large economic losses in barley-growing areas (Mathre, 1990). Although the production of conidia is expected to produce genetically identical clones, the high rate of appearance of new races with the ability to infect previously resistant varieties of barley suggests that *C. sativus* may have high mutation rates in avirulence genes, which determine race (Kumar *et al*., 2002).

General symptoms of SB include light brown lesions with whitish gray centers and chlorotic margins (Kumar *et al.,* 2002). Most varieties grown around the world are susceptible to *C. sativus*, although partial resistance has been reported (Arabi, 2005; Zhou and Steffenson, 2013). Genetic control of SB resistance is governed by quantitative traits. Two quantitative trait loci (QTLs) have been mapped to chromosomes 1S and 5S (Steffenson *et al.,* 1996). However, in the case of the host-specific toxin produced by *C. sativus,* the fungus which incites SB of barley, the toxin will produce all the symptoms characteristic of the disease; sensitivity to the toxin is correlated with susceptibility to the pathogen and toxin production by the pathogen is directly related to its ability to cause disease (Kumar *et al*., 2002).

Although the most effective control strategy for SB is cultivating resistant varieties, it has often achieved only short-term success due to the frequent breakdown of newly introduced resistance (Poudyal *et al*., 2005; Gontariu and Enea, 2012). This resistance breakdown has been attributed to genetic variability in *C. sativus* (Gilchrist *et al*., 1995). Different mechanisms have been suggested to explain the frequent generation of race variants, including heterokaryosis, parasexuality and mutations (Kumar *et al*., 2002; Arabi and Jawhar, 2007). However, the activity of the retrotransposon microsatellite amplified polymorphism (REMAP) is another possible mechanism that has been suggested which uses 1 LTR primer in combination with a primer designed for annealing at the 3' end of a stretch of a simple sequence repeat (SSR) and detects retrotransposons inserted near SSRs (Chadha and Gopalakrishna, 2005; Biswas *et al*., 2010).

Our overall question was how stable *C. sativus* isolates would be both genotypically and phenotypically during several serial transfers in one growing season. Therefore, the objective of this work was (i) to determine phenotypic variation of the two major pathotypes of *C. sativus* in Syria, Pt1 and Pt4 during seven serial transfers on barely plants and (ii) to investigate genetic variation of the isolates collected in each generation using DNA fingerprinting.

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2. Materials and Methods

Fungal isolates

For the inoculation process, two major pathotypes of *C. sativus* in Syria, Pt1 and Pt4, were used in this study. They were identical in spore morphology and colony colour, but differed widely in DNA patterns and virulence. After extensive greenhouse and laboratory screening over a 10-year period, Pt4 was proven to be the most virulent isolate to all barley genotypes available so far (Arabi and Jawhar, 2003; 2007), therefore it was used in this study. Each isolate was grown separately in 9 cm Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) and incubated for 10 days, at 22±1°C in the dark to allow mycelial growth.

Inocula preparation, serial transfer and isolation methods

After culturing Pt1 and Pt4 isolates on PDA medium, spores were collected by flooding each plate with 10 ml sterile water with 200 ppm of Tween 20, filtering through cheesecloth to remove mycelium and adjusting the concentration of the spore suspension to $2x10^4$ conidia/ml using hemacytometer counts of conidia. The universal susceptible control (cv. WI2291) plants from Australia were grown in pots filled with sterilized peatmoss, and arranged in a randomized complete block design with three replicates. Each experimental unit consisted of 10 seedlings. A full replicate consisted of 10 pots inoculated with Pt1 and Pt4 isolates. Pots were placed in a growth chamber at temperatures of 22±1°C (day) and 17±1°C (night) with a daylength of 12 h and a relative humidity (RH) of 80-90%. Plants were inoculated at growth stage (GS) 12 (Zadoks *et al*., 1974) by uniformly spraying each plant with 20 ml of conidial suspension with a hand-held sprayer. Plants were then placed in the dark at 95-100% R.H. for the first 18 h.

Pt1 and Pt4 isolates and single-conidial isolates from each of the three replicates from the 1st to 7th passage generations through barley plants were compared for virulence. Ten days after inoculation, one leaf per plant, for a total of three leaves per replicate, was sampled, surface sterilized and placed on a water agar plate. Three days later, a single conidium from each of the three plates was transferred to PDA for genotypic and phenotypic assays. The infection response based on the measurement of individual lesion size (dimension; mm) for each second leaf was assessed 10 days after inoculation according to Fetch and Steffenson (1999) scale. Each leaf was assessed separately and the assessments were performed by the same person in all experiments.

In vitro *phenotypic assays*

In vitro phenotypic assays were achieved by transferring plugs of mycelim (5 mm diameter) of each parent and generations onto PDA media and incubation at room temperature (22±1°C) in the dark. Conidial germination rate was recorded after 24 h on glass cover slips as described previously (Arabi and Jawhar, 2001). Colony diameter was measured seven days post incubation. Conidia were harvested from 15-day cultures using sterile distilled water and counted with a hemocytometer. All the experiments were repeated three times with five replicates, and a representative set of data is presented. Statistical analysis was performed using the STAT-ITCF program (Anonymous, 1988).

REMAP analysis

DNA extraction from parent and generation isolates was performed according to standard protocols (Leach *et al*., 1986). REMAP analysis and primer sequences were achieved using a standard method described by Kalendar *et al*. (1999). PCR reactions were performed in 25 µL reaction volume containing 1× *Taq* polymerase buffer (10 mmol Tris-HCl/L (pH 8.3), 50 mmol KCl/L, 2.5 mmol MgCl2/L, 0.01% gelatin), 0.5 U *Taq* polymerase (Eppendorf, Germany), 150 µmol of each dNTP/L, 0.4 µmol LTR1 primer/L, 0.6 µmol of ISSR primer/L (Table 1), and 50 ng of template DNA. A PCR was carried out in an Eppendorf DNA thermal master gradient cycler (Eppendorf Netheler-Hinz, Hamburg, Germany). The amplification conditions were as follows: 92°C for 5 min, followed by 40 cycles of 92°C for 45 s, 55°C for 45 s, and 72°C for 1 min; and a final extension step of 72°C for 10 min. Amplified products were electrophoresed in a 2% agarose gel using 1 × Tris-borate-EDTA buffer (100 mmol Tris-HCl/L, pH 8.3, 83 mmol boric acid/L, 1 mmol EDTA/L) at 100V. The gels were stained with ethidium bromide solution and visualized under ultraviolet illumination. Sizes of the amplified products were determined relative to a 100-bp DNA ladder (MBI Fermentas, York, UK).

3. Discussion and Conclusions

Disease symptoms (presence of necrosis and chlorosis) were severe on the susceptible genotype WI2291 that was infected with pathogenic isolates after 10 days of inoculation. Pt1 and their generations induced small round to oblong dark brown necrotic lesions, whereas, Pt4 and their generations induced solid dark brown necrotic lesions with expanding chlorosis (the 'classic' spot blotch lesion) in highly compatible interactions. As offspring isolates from the most diseased plants were serially passaged

Table 1 - REMAP primers used in the study

Primer no.	Sequence
1	(GA)8T+TGTTTCCCATGCGACGTTCCCCAACA
2	(AG)8T+GCATCAAAGGCATTGGAGGTG
3	(GA)8T+ GCATCAAAGGCATTGGAGGTG
4	(GA)8T+CACTAGTGATTCATTATGCTGAGTG
5	(AG)8T+GCATCAAAGGCATTGGAGGTG
6	(AG)8T+ CACTAGTGATTCATTATGCTGAGTG
7	(AG)8G+CCAATGGACTGGACATCCGATGGG
8	(AG)8T+ TGTTTCCCATGCGACGTTCCCCAACA
9	(AG)8G+ TGTTTCCCATGCGACGTTCCCCAACA

on barley, virulence of the aggressive isolate Pt4 was increased during seven transfers on plants, and the ability of weakly aggressive isolate Pt1 to maintain its virulence was observed (Table 2). All Pt4 isolates were highly virulent to cv. WI2291 with a mode of 4 (typifying >90% of the lesions observed on leaves), whereas, all Pt1 isolates were virulent exhibiting a mode of 2 (typifying >10% of the lesions observed on leaves), indicating that virulence was not significantly affected.

Infection responses of WI2291 to *C. sativus* isolates Pt1 and Pt4, and their generations are summarized in Table 2. The pattern of continuous incremental increase in virulence has been reported in *F. oxysporum* f. sp. *ciceris*, on chickpea (Jiménez-Gasco *et al*., 2004), and in *Magnaporthe oryzae* on rice (Park *et al*., 2010). However, the pathosystems involving the genera of *Cochliobolus* and the involvement of host-specific toxins in pathogenicity and virulence are well documented (Olbe *et al*., 1995).

The number of SB lesions (presence of necrosis and chlorosis) were always high in the virulent isolate Pt4 during the serial passages (Fig. 1). This can be attributed to a higher proportion of Pt4 spores being able to establish lesions than Pt1 spores. This indicates that the more virulent Pt4 causes more lesions (per leaf) from a given inoculum dose than Pt1 as presented in figure 1. The existence of pathotypes expressing differential virulence on host genotypes is uncommon in species related to *C. sativus*. Differential reactions, such as those expressed by the pathotypes Pt1 and Pt4 on two-rowed genotypes (WI 2291), usually are a feature of gene-for-gene interactions (Flor, 1956) or an incompatibility system (Briggs and Johal, 1994). A hypothesis that these two differentially virulent *C. sativus* isolates are pathotypes that produce two different types of host-specific toxins may also be valid.

Additionally, no significant differences were observed between the parental isolates and any isolates derived from them through the seven serial transfers for any of the phenotypic characters tested *in vitro* including mycelial growth, conidiation and conidial germination on PDA media (Fig. 2). These results are similar to those of Latterel and Rossi (1986), who reported no changes in cultural characteristics in *Magnaporthe oryzae* isolates after serial transfers of the same isolates from stock cultures over a long period of time.

To evaluate genotypic stability during serial transfers, REMAP fingerprinting technique was used. The REMAP haplotypes of seven generations were identical to their parent isolates (Fig. 3); this might be an indicator of the stability of transposable elements during serial passages. The lack of molecular variation in offspring of Pt4 isolates with increased virulence (Table 2; Fig. 3) is consistent with the fact that mutation rates at virulence loci are higher than those at the molecular loci that define genotypes (Goodwin *et al*., 1995).

The results of this study demonstrate that the virulence of the offspring isolates generated from the aggressive isolate Pt4 significantly increased after seven successive

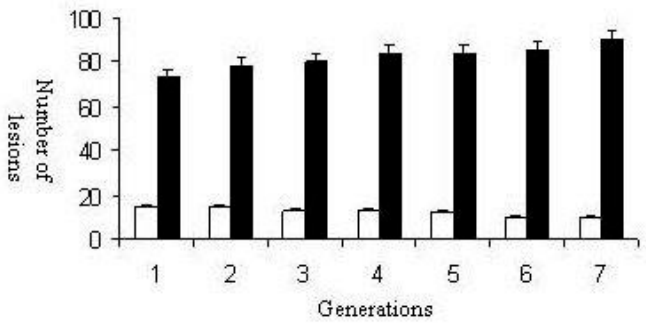

Fig. 1 - Number of lesions caused by Pt1 and Pt4 isolates after seven serial transfers barley cv. WI2291.

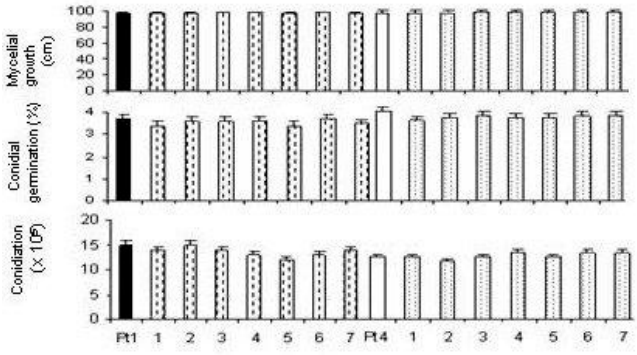

Fig. 2 - Cultural characterization of two *C. sativus* isolates Pt1 and Pt4 after seven passages on PDA medium and through barley plants.

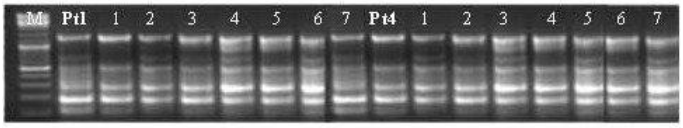

Fig. 3 - Agarose gel electrophoresis of REMAP analysis Pt1 and Pt4 parent isolates after seven passages through barley cv. WI2291 using primer; (GA)8T+GCATCAAAGGCATTGGAGGTG. M–marker ladder 1Kb.

Table 2 - Infection responses of barley cv. WI 2291 infected with parents and progeny isolates of *Cochliobolus sativus* based on the scale of Fetch and Steffenson (1999)

Isolates	Infection response	Parent	Passages						
			1	2	3	4	5	6	7
Pt1	Mode [z]	2	1	2	2	2	2	1	1
	Range [y]	1-2	1-2	2	1-2	1-2	1-2	1-2	1-2
Pt4	Mode	4	4	4	4	4-5	4	4	4-5
	Range	7-9	6	7	7-8	8-9	9	9	9

[z] Mode= The most common infection response observed on the barley cv. WI2291.

[y] Range= The lowest and highest infection responses observed on the barley cv. WI2291.

passages, in contrast to the lack of significant changes in those obtained from the weakly aggressive isolate Pt1. No apparent differences in phenotypes, including mycelial growth, conidiation and conidial germination, were observed among isolates from the same parent isolate on artificial medium. All single-conidial of the parents and their generations were stable genotypically during the serial transfers with a change in virulence of the aggressive isolate generations.

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Genetic divergence studies regarding different growth and foliage characters of walnut (*Juglans regia* L.) germplasm

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Key words: divergence, exotic cultivars, growth, local selections, walnut germplasm.

Abstract: Genetic divergence of 29 exotic walnut cultivars introduced from abroad and 25 local selections from seedling population was studied. The analysis of variance revealed significant differences among accessions for each character under study. Based on Mahalanobis D^2 values the accessions were grouped into eight clusters. Cluster I had a maximum of 12 accessions followed by cluster II with 11 accessions. The mean intra and inter cluster distance (D) revealed that cluster VII had the highest intra cluster distance (2.171), while the inter cluster distance was the greatest between cluster II and IV (10.528). Characters like plant height, trunk girth, plant spread, plant volume, leaf length, leaf width and number of leaflets contributed the most to total divergence.

1. Introduction

Persian walnut (*Juglans regia* L.) is one of the most important nut crops grown in temperate regions and it produces edible nuts of high nutritional value. In India there are no systematic orchards of walnut containing standard cultivars. Although some efforts have been made in this direction in the last two decades and the superior selections from the seedling populations were selected through field surveys in different walnut growing districts of Himachal Pradesh (Thakur, 1993; Gupta, 1999; Sharma, 1999, 2002; Thakur *et al*., 2005). However, a large number of exotic accessions have also been introduced from abroad and a germplasm collection block has been maintained after propagation of these selections and exotic accessions on the seedling rootstock. Therefore characterization and evaluation of this germplasm is important for future use and a wider divergence is a prerequisite for breeding purposes. Information about the extent of genetic divergence is critical for the improvement of any crop in order to have heterotic responses and desirable segregants. Furthermore, information on the nature and degree of genetic divergence present in the collected germplasm could help for further improvement through hybridization.

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2. Materials and Methods

The present investigation was undertaken on three- to four-year-old plants for a total of 54 indigenous selections collected through field survey at different locations in Himachal Pradesh and exotic cultivars of Persian walnut introduced directly from abroad (Table 1). These were propagated on seedling rootstocks and are presently growing at Oachghat Block of Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni-Solan (Himachal Pradesh) located around 1275 m above mean sea level and between 31°N latitude and 77°E longitude. Data for various growth [plant height (m), trunk girth (cm^2), plant spread (cm), TCSA (cm^2) and plant volume (cm^3)] and foliage characters [leaf length (cm), leaf width (cm), leaf area (cm^2), leaflet length (cm), leaflet width (cm) and number of leaflets] were recorded. Ten compound leaves were taken from each individual tree for foliage characters and average means were calculated. Standard procedures were followed to calculate the mean, standard deviation and coefficient of variation for various characters as described by Panse and Sukhatme (1995). The genetic divergence among the accessions was estimated by Mahalanobis D^2 statistic as suggested by Rao (1952).

Table 1 - Different exotic and indigenous walnut accessions and their place of origin

S. No.	Accession	Place of origin
Exotic cultivars		
1.	Adams 10	USA
2.	Aksu≠24	China
3.	Aksu≠71	China
4.	Aksu≠81	China
5.	Aksu≠210	China
6.	Aksu≠417	China
7.	Aksu Hun ≠85	China
8.	Bulgaria 3	Bulgaria
9.	Chandler	USA
10.	Chico	USA
11.	Cisco	USA
12.	Conway Mayette	USA
13.	Corne	France
14.	Graves Franquette	USA
15.	Hotien 8	China
16.	Howard	USA
17.	Howe	USA
18.	Mayette	USA
19.	Meylannaise	France
20.	Nn 88 Godyn	Poland
21.	Parisienne	France
22.	Placentia	USA
23.	Ronde de Montignac	France
24.	Scharsch Franquette	USA
25.	Serr	USA
26.	Shinrei	China
27.	Xin Zad Fen	China
28.	Xin Zheng Zhu	China
29.	Zhong Lin≠3	China
Indigenous selections		
30.	Brij Lal Selection	H.P. (India)
31.	Chamba Selection-20	H.P. (India)
32.	Chamba Selection-32	H.P. (India)
33.	Chamba Selection-60	H.P. (India)
34.	Chamba Selection-101	H.P. (India)
35.	Chamba Selection-123	H.P. (India)
36.	Daulat Ram Selection	H.P. (India)
37.	Jaunaji Selection -2	H.P. (India)
38.	Jaunaji Selection –4	H.P. (India)
39.	Jaunaji Selection –6	H.P. (India)
40.	Jaunaji Selection-7	H.P. (India)
41.	Jaunaji Selection-10	H.P. (India)
42.	Jaunaji Selection-12	H.P. (India)
43.	Jogindernagar Selection-23	H.P.(India)
44.	Jogindernagar Selection-39	H.P. (India)
45.	Jogindernagar Selection-61	H.P. (India)
46.	Jogindernagar Selection-122	H.P. (India)
47.	Nauradhar Selection-10	H.P. (India)
48.	Nauradhar Selection-36	H.P. (India)
49.	Nauradhar Selection-53	H.P. (India)
50.	Rajgarh Selection-1	H.P. (India)
51.	Rajgarh Selection-8	H.P. (India)
52.	Rajgarh Selection-11	H.P. (India)
53.	Selection No. 8	H.P. (India)
54.	Selection No. 51	H.P. (India)

All the accessions were grouped into clusters according to the Tocher's method described in Rao (1952).

3. Results and Discussion

The analysis of variance revealed significant differences among the genotypes for each character, indicating the existence of variability among the genotypes for such characters. On the basis of the relative magnitude of D^2 values, 54 accessions were grouped, using Tocher's method, into eight clusters. Cluster I was the largest with 12 accessions followed by cluster II having 11 accessions, and cluster V having 10 accessions; clusters III, IV, VI, VII, VIII had 5, 1, 9, 4, 2 accessions, respectively (Fig. 1 and Table 2). The highest intra cluster distance was registered in cluster VII (2.171) followed by cluster II (1.951), while the minimum intra cluster value was exhibited by cluster IV (0.000). The minimum inter-cluster D^2 value was recorded between cluster I and V (1.821), indicating a close relationship and similarity of most traits of the accessions. Hence, selection of parents from these clusters should be avoided. However, the highest inter-cluster D^2 value was observed between clusters II and IV (10.528), followed by clusters I and IV (10.411), confirming a wide genetic distance between these groups (Table 3). Since these clusters exhibited the greatest inter-cluster distances, selection of parents from such clusters for hybridization programmes could help to develop novel hybrids. A similar trend of clustering patterns has been reported by Barua and Sharma (2003) in apple, Thakur *et al*. (2005) in almond., Kaushal and Sharma (2005) in pecan, and Sharma and Sharma (2005), Pandey and Tripathi (2007), and Sharma *et al*. (2010) in walnut.

The mean value, standard deviation and co-efficient of variability on various growth and foliage traits of cluster is presented in Table 4. Cluster IV had the highest mean values for characters plant height (5.50 m), trunk girth (33.00 cm) and TCSA (86.55 cm^2), while cluster VIII exhibited the highest mean values for plant spread (110.00 cm), plant volume (1.71 cm^3), leaf length (56.50 cm), leaf width (30.25 cm), leaf area (57.35 cm^2), leaflet length (16.17 cm). Maximum mean values for leaflet width (7.19 cm) and number of leaflets (9.73) was recorded in clusters VI and II, respectively. Clusters IV and VIII can be expected to give promising and desirable recombinations in segregating generations because they comprise desirable features as revealed from their cluster means. Similarly, maximum co-efficient of variability for plant height (40.00%), trunk girth (33.11%), plant spread (42.08%), TCSA (65.21%), plant volume (87.50%) and number of leaflets (23.12%) was observed in cluster II, however, cluster I had the highest co-efficient of variability for traits leaf area (32.86%), leaflet length (13.46%) and leaflet width (19.79%).

In the principal component analysis presented in Table 5, the first vector shows the highest eigen value (4.332)

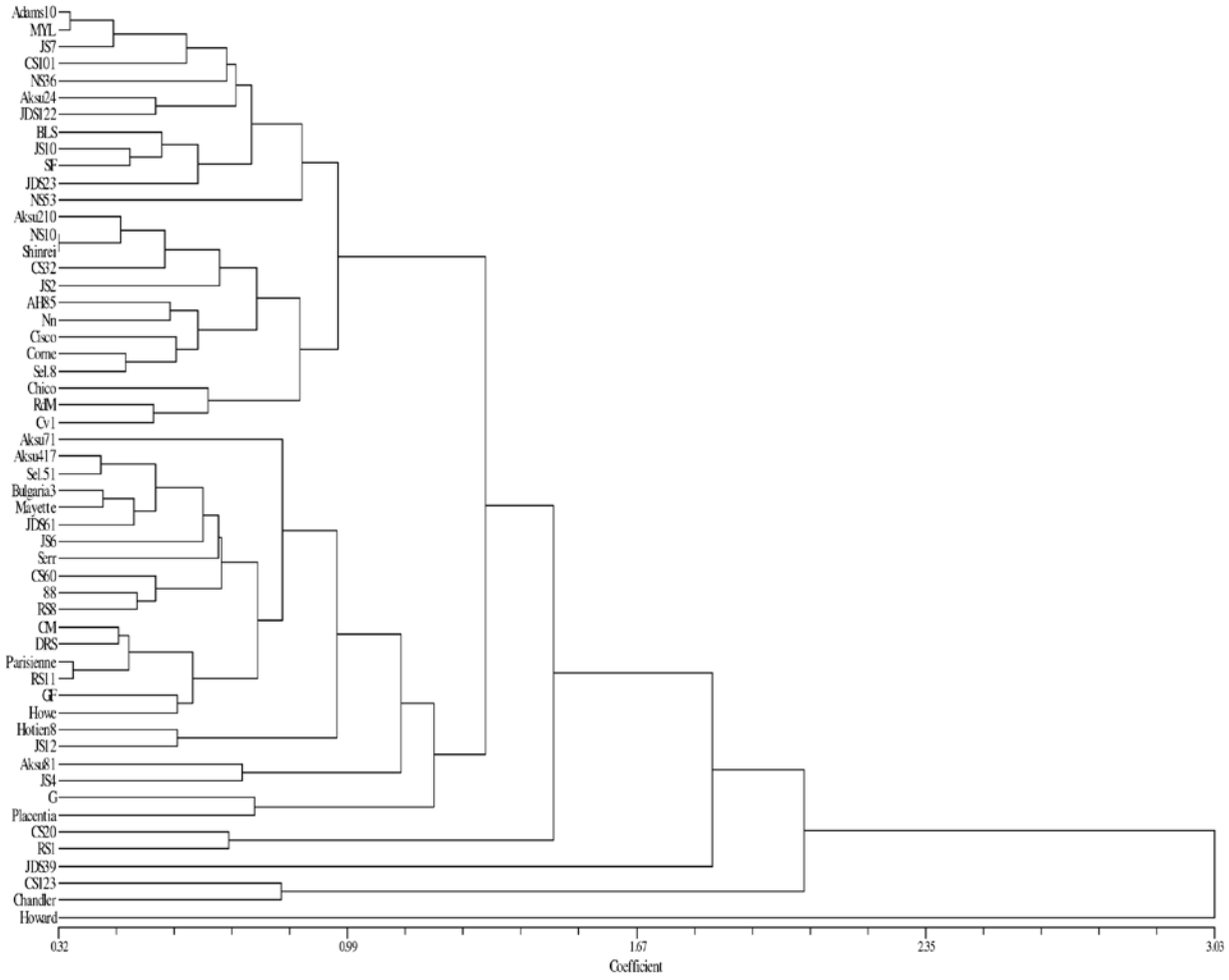

Fig. 1 - Dendrogram showing different clusters of genotypes, grouped by Tocher's method, in walnut accessions.

and accounts for 39.38% of the total variation. The first vector is the combination of leaf length, trunk girth, plant height and leaflet length. The second vector has an eigen value of 3.008 and explains 27.34% of total variation, and this factor is mainly the combination of number of leaflets and trunk girth. The third vector has an eigen value of 1.155 and total variation of 10.50%, from the combination of leaf width and plant spread. The fourth vector has an eigen value of 1.047 and total variation of 9.52%, with the maximum contribution of trunk girth. Vector fifth has an eigen value of 0.424 and total variation of 3.85%, mainly a combination of plant volume and plant spread. The sixth vector has an eigen value of 0.347 with total variation of 3.15 % and the highest contribu-

Table 2 - Distribution of 54 walnut accessions into various clusters

Clusters	Number of accessions	Accessions
I	12	Aksu≠210, Chamba Selection-32, Chamba Selection-60, Conway Mayette, Hotien 8, Jaunaji Selection-2, Jaunaji Selection-12, Jogindernagar Selection-61, Nauradhar Selection-10, Parisienne, Ronde de Montignac, Xin Zheng Zhu
II	11	Adams 10, Aksu≠24, Chamba Selection-101, Jaunaji Selection-7, Jaunaji Selection-10, Jogindernagar Selection-39, Jogindernagar Selection-122, Meylannaise, Nauradhar Selection-36, Nauradhar Selection-53, Selection No. 8.
III	5	Aksu≠81, Cisco, Daulat Ram Selection, Jaunaji Selection-4, Scharsch Franquette
IV	1	Howard
V	10	Aksu≠71, Aksu≠417, Bulgaria 3, Graves Franquette, Howe, Jaunaji Selection-6, Mayette, Rajgarh Selection-1, Selection No. 51, Xin Zad Fen
VI	9	Aksu Hun≠85, Brij Lal Selection, Chico, Corne, Jogindernagar Selection-23, Nn 88 Godyn, Serr, Shinrei, Zhong Lin ≠3
VII	4	Chamba Selection-20, Placentia, Rajgarh Selection-8, Rajgarh Selection-11
VIII	2	Chamba Selection-123, Chandler

Table 3 - Average inter- and intra-cluster distance among various clusters in walnut germplasm

Clusters	I	II	III	IV	V	VI	VII	VIII
I	**1.658**							
II	3.535	**1.951**						
III	2.295	3.975	**1.497**					
IV	10.411	10.528	8.795	**0.000**				
V	1.821	4.953	2.505	10.048	**1.396**			
VI	3.159	2.115	2.689	9.396	3.875	**1.657**		
VII	3.870	4.898	2.984	9.037	3.536	3.388	**2.171**	
VIII	7.505	5.835	6.356	8.920	7.931	5.081	5.220	**1.373**

Bold figures represent intra-cluster distances.

Table 4 - Mean, standard deviation and coefficient of variation for various clusters on the basis of growth and foliage characters of walnut germplasm

S. No.	Characters	Parameters	Clusters							
			I	II	III	IV	V	VI	VII	VIII
1	Plant height (m)	Mean	1.12	1.00	2.98	5.50	1.36	1.77	1.91	2.65
		SD	0.27	0.40	0.85	0.00	0.52	0.49	0.61	0.21
		COV	24.11	40.00	28.52	0.00	38.23	27.68	31.94	7.92
2	Trunk girth (cm)	Mean	7.15	7.55	10.40	33.00	9.45	10.75	12.12	16.00
		SD	2.05	2.50	2.25	0.00	2.60	1.70	3.12	2.83
		COV	28.67	33.11	21.63	0.00	27.51	15.81	25.74	17.69
3	Plant spread (cm)	Mean	31.71	36.36	44.00	50.00	39.50	51.94	70.00	110.00
		SD	11.48	15.30	13.87	0.00	12.95	17.36	17.80	14.14
		COV	36.20	42.08	31.52	0.00	32.78	33.42	25.43	12.85
4	TCSA (cm^2)	Mean	4.38	5.03	8.91	86.55	7.61	8.27	12.28	20.74
		SD	2.17	3.28	3.29	0.00	4.56	2.99	6.10	7.25
		COV	49.54	65.21	36.92	0.00	59.92	36.15	49.67	34.96
5	Plant volume (cm^3)	Mean	0.07	0.08	0.32	0.72	0.15	0.30	1.57	1.71
		SD	0.06	0.07	0.21	0.00	0.11	0.25	0.76	0.57
		COV	85.71	87.50	65.62	0.00	73.33	83.33	48.41	33.33
6	Leaf length (cm)	Mean	33.46	46.73	35.00	40.00	27.05	39.33	32.62	56.50
		SD	5.47	3.98	2.89	0.00	3.46	4.84	6.94	6.36
		COV	16.35	8.52	8.26	0.00	12.79	12.31	21.27	11.26
7	Leaf width(cm)	Mean	18.55	24.97	18.95	20.50	15.50	22.58	18.06	30.25
		SD	2.90	2.26	2.15	0.00	2.41	3.97	1.88	0.35
		COV	15.63	9.05	11.34	0.00	15.55	17.58	10.41	1.16
8	Leaf area (cm^2)	Mean	29.73	56.69	33.34	38.39	26.28	48.00	38.10	57.35
		SD	9.77	8.90	8.12	0.00	6.25	8.64	9.60	2.96
		COV	32.86	15.70	24.35	0.00	23.78	18.00	25.19	5.16
9	Leaflet length (cm)	Mean	10.70	14.57	10.79	12.50	8.81	13.58	10.64	16.17
		SD	1.44	1.15	1.02	0.00	1.13	1.56	1.32	0.23
		COV	13.46	7.89	9.45	0.00	12.83	11.49	12.41	1.42
10	Leaflet width (cm)	Mean	4.95	6.93	5.21	5.46	4.55	7.19	5.58	7.00
		SD	0.98	0.68	0.82	0.00	0.65	0.55	1.05	0.00
		COV	19.79	9.81	15.74	0.00	14.28	7.65	18.82	0.00
11	Number of leaflets	Mean	9.29	9.73	8.80	9.00	7.65	8.17	8.00	8.00
		SD	1.32	2.25	0.45	0.00	0.82	0.87	1.15	1.41
		COV	14.21	23.12	5.11	0.00	10.72	10.65	14.37	17.62

Table 5 - Eigen vectors, eigen roots and percent variation explained by growth and foliage characters of walnut germplasm

S. No.	Characters	Eigen roots	Per cent variation	Eigen vectors										
				1	2	3	4	5	6	7	8	9	10	11
1	Plant height (m)	4.332	39.38	0.131	0.180	0.239	0.147	0.192	0.415	0.419	0.404	0.432	0.369	0.035
2	Trunk girth (cm)	3.008	27.34	0.458	0.490	0.293	0.456	0.295	-0.145	-0.126	-0.195	-0.169	-0.196	-0.163
3	Plant spread (cm)	1.155	10.50	-0.202	-0.228	0.424	-0.383	0.496	-0.128	0.018	-0.037	-0.042	0.068	-0.557
4	TCSA (cm^2)	1.047	9.52	-0.059	-0.132	0.371	-0.170	0.326	0.211	0.099	-0.153	-0.129	-0.354	0.697
5	Plant volume (cm^3)	0.424	3.85	-0.006	-0.018	-0.379	0.051	0.579	-0.023	-0.483	0.308	-0.160	0.337	0.225
6	Leaf length (cm)	0.347	3.15	0.818	-0.293	-0.075	-0.391	-0.025	-0.076	0.057	-0.158	0.037	0.213	0.066
7	Leaf width(cm)	0.243	2.20	-0.054	0.125	0.512	-0.070	-0.326	-0.468	-0.111	0.276	-0.231	0.418	0.270
8	Leaf area (cm^2)	0.191	1.74	-0.199	0.026	-0.206	0.179	0.242	-0.426	0.469	-0.506	0.101	0.357	0.180
9	Leaflet length (cm)	0.118	1.07	0.110	-0.047	-0.209	-0.024	0.141	-0.452	0.446	0.565	-0.110	-0.432	-0.002
10	Leaflet width (cm)	0.091	0.83	-0.013	0.060	0.073	-0.090	0.049	-0.362	-0.354	0.001	0.813	-0.217	0.126
11	Number of leaflets	0.045	0.41	-0.060	0.742	-0.192	-0.627	0.024	0.058	0.074	-0.056	-0.056	-0.003	0.021

Percent variation explained by first 7 components = 95.9517

tion came from plant height, while the minimum eigen roots value 0.045 and percent contribution towards diversity (0.41%) was observed for the eleventh component, with a maximum contribution from TCSA. The percent variation explained by the first seven components was 95.95 (Table 5).

The genetic diversity among genotypes could be due to various factors, like heterogeneity, genetic architecture of populations and developmental traits, as described by (Murty and Arunachalam, 1966). Rao *et al.* (2003) reported that geographical distribution and genetic diversity are correlated and concluded that eco-geographically different cultivars also differ from each other genetically. A clustering pattern similar to our pattern was reported in walnut (Pandey and Tripathi, 2007) and hazelnut (Srivastava *et al*., 2010). Sardana *et al.* (1997) observed that cluster means reveal the inner diversity in the material under study. De *et al.* (1988) proposed that traits contributing the most towards the D^2 values should be given priority in choosing a cluster for further selection and choice of parents for hybridization.

Based on the present findings of genetic divergence and its component analysis it can be concluded that intercrossing between genotypes of genetically diverse clusters that show a superior mean outcome may be helpful for obtaining desirable segregates. In the case of walnut germplasm the highest genetic diversity was registered between clusters II and IV. Comparison of the cluster means for 11 characters indicated that the studied traits considerably differed between the clusters. Therefore, it is suggested to cross 'Howard' with 'Chamba Selection 123' and with 'Chandler' as these accessions are among the most distant clusters, with a high mean outcome, in order to get desirable transgressive segregates.

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Enzymatic activity of the endophytic *Fusarium* species strains isolated from wheat

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Key words: *Fusarium* spp., hydrolytic enzymes, wheat.

Abstract: *Fusarium* is a genus of fungi that cause some of the most important plant diseases affecting agricultural and horticultural crops. Members of the genus establish an endophytic role inside the tissue of plants and produce a wide range of biologically active metabolites and enzymes. In the present study, the enzymatic profiles of several dominant *Fusarium* spp. were determined under solid state fermentation and activities were detected for xylanase, lipase, amylase, polygalacturonase, filterpase, and carboxy-methyl cellulase. Each *Fusarium* spp. showed a wide range of enzyme activities and protein contents. The ability to produce these enzymes was distributed amongst the strains tested, however amylase and xylanase *F. solani* Sy7 was found in a high percentage of strains. This study provides additional information to support future research about the industrial potential of these enzyme-producing species.

1. Introduction

Fusarium is an endophytic genus consisting of an array of species responsible for damping-off, root rot, and vascular wilt in a multitude of economically important plant species (Summerell *et al.*, 2001). The importance of *Fusarium* spp. in the current context is that infection may sometimes occur in developing seeds (Tajehmiri *et al.,* 2014). Because of this unusually wide range of symptoms, cell wall degrading enzymes (CWDEs) could have a range of important functions for the fungus in penetration, in heads, and in saprotrophic growth in dead tissue. This ecological habitat of the fungus implies that *Fusarium* could be a useful resource of extracellular enzymes (Kwon *et al.,* 2007; Bakri *et al.,* 2013).

Extracellular enzymes are thought to be particularly important CWDEs in interactions between *Fusarium* spp. and their hosts (Ahmed *et al.,* 2012). Much research work is still needed to fully understand the degradation process, and particularly the enzymes and other metabolites secreted by the fungi during infection. Information on these aspects could help to elucidate the biochemical mechanisms of wheat infection by *Fusarium* diseases and consequently facilitate fungal strain selection for industrial applications. However, studies of enzyme production by a phytopathogenic fungus are complicated by the presence of the plant, particularly by the presence of plant enzymes and microbial enzyme inhibitors that occur in plants. The most practical way to study the production of enzymes by a fungus is therefore to study the production of its enzymes on artificial growth media that contain no plant or enzyme inhibitors.

Solid-state fermentation (SSF) is considered an attractive alternative method for the production of industrially demanded enzymes that employ microorganisms (Krishna, 2005). SSF was defined by Pandey (2003) as a fermentation process involving solids in the absence (or near absence) of free water. Among the microorganisms that are capable of growing on solid substrates, only filamentous fungi can grow to a significant extent in the absence of free water (Guimaraes *et al.,* 2006).

The present study was undertaken to assess the potentialities of several *Fusarium* spp. in the production of industrially relevant enzymes under SSF conditions.

2. Materials and Methods

Isolation of Fusarium *species*

Fusarium spp. have been isolated from infected wheat seeds showing disease symptoms, and 21 were screened out of 105 strains for their host-pathogen interactions (Alazem, 2007). Seeds were sterilized to remove all microbial epiphytes by soaking them in 1:5 dilutions of NaOCl (sodium hypochlorite) solution for 15 min. They were then rinsed in sterile distilled water and dipped in 70% ethanol for 10 min. Subsequently, the seeds were washed with distilled water, dried between sterilized filter paper, and incubated in 9-cm Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) for 10 days, at 23±1°C in the dark to allow mycelial growth. All strains were identified morphologically according to Nelson *et al.*

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(1983). The *Fusarium* spp. strains used in the study are listed in Table 1. The cultures were maintained on silica gel at 4°C until needed.

Extraction of enzymes from solid-state cultures

Enzyme production by the *Fusarium* spp. strains was carried out in 250 ml Erlenmeyer flasks containing 5 g of solid substrate and nutrients (based on 100 ml of liquid medium) plus distilled water to adjust the moisture content to 75%. Fresh fungal spores were used as inoculums and 1 ml spore suspension (containing around 10^6 spores/ml) was added to sterilized medium and incubated at 30°C. Flasks were removed after cultivation and the enzyme was extracted by adding distilled water containing 0.1% Triton x 100 to make the in-flask volume equivalent to 100 ml. Flask contents were stirred for 1.5 h on a magnetic stirrer. The clear supernatant was obtained by centrifugation (5000 x *g* for 15 min) followed by filtration (Whatman no. 1. paper).

Carboxy methyl-cellulase and filter paperase (Fpase) activity

Extra cellular enzymes were extracted by filtering the culture through Whatman No. 1 filter paper. The CMCase and Fpase activity were measured using the methods described by Refaz *et al.* (2013). One unit of enzyme activity (IU) was defined as the amount of enzyme that released 1 µmol of glucose per ml per minute.

Amylolytic activity

A-amylase activity was determined as described by Okolo *et al.* (2001). The reaction mixture consisted of 1.25 ml of 1% soluble starch, 0.25 of 0.1 M acetate buffer (pH5.0), 0.25 ml distilled water and 0.25 ml of crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalent) were estimated by the dinitrosalicylic acid method of Miller (1959). One unit (IU) of α-amylase is defined as the amount of enzyme that releases 1 µmol of glucose equivalent per min under the assay conditions.

Lipolytic activity

Lipase activity was determined using 1 ml sunflower oil, 5 ml of 50 mM phosphate buffer pH 7.0 and 1 ml enzyme solution. The assay was carried out according to the method of Park *et al*. (1988). One unit of lipase activity was defined as the amount of enzyme liberating 1µ mol of fatty acid per min under the experimental conditions.

Pectinolytic activity

Assay of polygalacturonase (PGase) activity was carried out according to Marcia *et al.* (1999). PG activity was determined by measuring the release of reducing groups using the dinitrosalicylic acid reagent (DNS) assay (Miller, 1959). The reaction mixture containing 0.8 ml of 1% citric pectin with 67% of metoxilation in 0.2M citrate-phosphate, pH 6.0 buffer and 0.2 ml of culture superna-

Table 1 - Enzyme activity (U/g) of the 21 strains of *Fusarium* species used in this study

Strain	Xylanase	carboxy-methyl cellulase	Fpase	Polygalacturonase	Amylase	Lipase
F.culmorum SY1	20.3	3.31	2.46	23.52	45.5	61.28
2	96.36	6.67	3.64	38.72	55.36	0.30
3	163.69	3.89	2.03	70.96	54.6	0.90
6	131.93	2.78	2.03	80.4	51.13	117.04
9	12.16	1.76	2.03	15.68	52	65.04
12	115.92	2.32	2.03	74.24	66.8	81.28
13	90.64	2.87	2.03	23.92	47.4	0.50
14	19.52	2.09	2.03	41.04	51.4	0.10
F.solani SY7	757.2	5.27	2.03	67.92	118.35	81.28
11	112.16	2.66	2.03	22.72	40.4	0.70
20	234.96	1.43	2.03	61.04	39.25	0.90
35	125.6	2.39	2.03	43.76	41.7	57.52
F.verticillioides SY15	61.92	1.79	2.03	32	44.8	0.40
16	16.56	3.01	2.03	31.44	54.3	0.20
19	108.56	5.74	2.03	97.92	70.95	87.6
27	129.92	8.92	2.03	35.52	76.13	73.76
29	138.72	2.47	2.03	80	38.7	60.4
31	151.92	2.14	2.03	41.52	43.5	54.8
F.equiseti SY41	93.2	2.41	2.03	29.52	33.6	0.60
42	84.64	4.92	2.03	20.56	45.1	0.40
43	122.24	0	2.03	68	58.8	82.48

LSD= Least Significant Difference at $P < 0.05$.

tant, was incubated at 40ºC for 10 min. One unit of enzymatic activity (U) was defined as 1 µmol of galacturonic acid release per minute.

Xylanolytic activity

Xylanase activity was measured with the optimized method described by Bailey *et al.* (1992), using 1% birchwood xylan as substrate. The solution of xylan and the enzyme at appropriate dilution were incubated at 55°C for 5 min and the reducing sugars were determined by the dinitrosalicylic acid method described by Miller (1959), with xylose as standard. The released xylose was measured spectrophotometrically at 540 nm. One unit of xylanase is defined as the amount of enzyme required to release 1 µmol of reducing sugar as xylose equivalent per min under the above assay conditions.

Protein determination

Total proteins were determined for their importance when purifying an enzyme, since purity depends on the removal of unwanted proteins, and can be assessed by relating the activity to total protein present. The protein content in the enzyme preparation was determined according to the method of Lowry *et al*. (1951).

Statistical analysis

All the experiments were performed in triplicate and the means were analyzed statistically with the analysis of variance (Anonymous, 1988) using the STAT-ITCF computer package to test for differences in enzyme production among *Fusarium* spp. strains.

3. Results

All 21 strains of endophytic *Fusarium* spp. tested were able to produce one or the other extracellular enzymes (Table 1); some of the strains were able to produce all six enzymes tested. The profiles of extracellular enzymatic activities varied among *Fusarium* spp., but some general features were noted. Significant differences ($P<0.05$) in the mean yield values were detected among strains, with high values being consistently higher in the strain *F. solani7* for xylanase and amylase activities with mean values of 757.2 and 118.35 U/g, respectively. Some authors have suggested that the absence of catabolic repression in SSF systems is due to several factors collectively, including the slow and low processes of diffusion in solid state cultures due to low water activity (Krishna, 2005).

On the other hand, CMCase and Fpase activity were detected in the lowest quantity, in terms of total units per culture (Table 1). Whereas, *F. verticillioides* strain *SY19*, showed the highest PGase activity with a mean value 97.92 U/g. Hoondal *et al.* (2002) reported that degradation of host tissue by phytopathogens generally begins with the production of pectinolytic enzymes, which are the major enzymes involved in plant attack. The effect of different carbon sources on pectinase synthesis by fungi in SSF have been studied and it is generally agreed that the optimum medium for the enhanced production of extracellular pectinase contains pectic materials as an inducer (Yadav *et al.,* 2005).

Additionally, the results show that *Fusarium* spp. strains varied in lipase activity (Table 1), which could be related to a differential capacity to infect wheat. Pritsch *et al.* (2000) suggested that lipases might have participated, to a certain extent, in prior degradation of the cuticle. On the other hand, protein contents also showed significant ($P<0.05$) differences among *Fusarium* spp. (Fig. 1). The increase in protein content in some strains - *F. solani Sy7*, *F. culmorumSy2* and *F. verticillioides Sy27* - that produced high enzymatic activity suggests the growth of fungi contributing to the fungal protein by utilizing available nutrients in the substrate, making it possible to metabolize the available protein in the substrate. Consumption of substrate protein the production of biomass protein are reported by Scopes (1993).

4. Discussion and Conclusions

The *Fusarium* spp. strains studied here produced significant levels of enzyme activity *in vitro.* The results might indicate that this activity influences the aggressiveness of the strains of *Fusarium* spp. towards wheat plants. Kang and Buchenauer (2000) showed that *Fusarium culmorum* infects the wheat ovary usually through the junctions between the epidermal cell walls. These junctions may be a more preferable site for entry of the pathogen, allowing a quicker establishment of infection. However, Priest (1984) showed that there are several possible regulatory mechanisms in enzyme production including induction. The action of such enzymes gives rise to the possibility that the "genetic recombination" of the endophyte with the host which occur in evolutionary time. This may be the reason why some endophytes *Fusarium* spp. can produce some photochemicals originally characteristic of the host.

Promputtha *et al.* (2007) provided phylogenetic evidence indicating that the endophytes produced the same degrading enzymes as their saprobic counterparts. However, the capability of studied endophytes, such as the *Fusarium spp.* in this work, to produce different enzymes should have an important role as saprobes since they do not decompose the host living tissue. These degrading en-

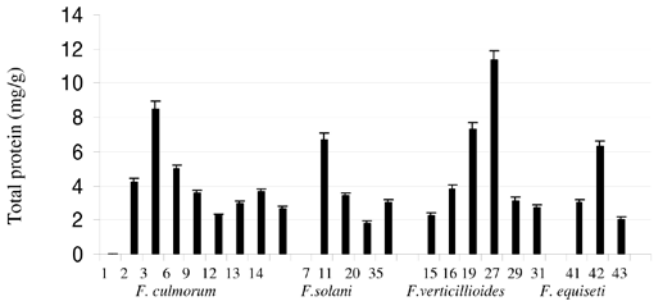

Fig. 1 - Total protein of *Fusarium* spp. strains under SSF.

zymes are important factors which affect the lifestyle of these species to become pathogens on wheat plants. Indeed, *Fusarium* species strains produced *in vitro* enzymes, a good indication that they may also do so under natural conditions. Schwarz *et al.* (2002) reported that *Fusarium* can produce various CWDEs *in vitro* and analyzed their regulation, suggesting that the initial infection depends of the secretion of these enzymes.

The present study demonstrates that *Fusarium* spp. are able to produce quite a good source of different types of industrially important enzymes. Knowledge of the types and amounts of enzymes produced by these species would be useful for the selection of strains best suited for industrial requirements. There are currently no reported studies on xylanase, lipase, amylase, polygalacturonase, filterpase, and carboxy-methyl cellulase enzymes to the authors' knowledge and thus there is a need for further in-depth studies on these isolated bioactive *Fusarium* spp. strains.

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Impact of UV irradiation in leaves, fruits and suspension-cultured cells of Micro-Tom, tomato

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Key words: ethylene, pathogenesis, redox, ripeness, salicylic acid, *Solanum lycopersicum*, ultraviolet.

Abbreviations: ACC= 1-amincocyclopropane-1-carboxylic acid; *ACS1a*= 1-amincocyclopropane-1-carboxylic acid synthase; APX= cytosolic ascorbate peroxidise; CIA= chloroform and isoamyl alcohol; DNase= deoxyribonuclease; PAL= phenylalanine ammonia-lyase; PR= pathogenesis-related; ROS= reactive oxygen species; RT-PCR= reverse transcription polymerase chain reaction; SA= salicylic acid; UV, ultraviolet.

Abstract: The present study aims to understand both positive and negative impacts of ultraviolet (UV) rays in living dwarf tomato plants (*Solanum lycopersicum* L. *cv*. Micro-Tom). This paper examines the impact of UV-C (254 nm) and UV-A (365 nm) on induction of cell death and expression patterns of pathogenesis-related (PR), stress-related and redox-related genes, namely, of 1-amincocyclopropane-1-carboxylic acid synthase (*ACS1a*), cytosolic ascorbate peroxidise (APX), phenylalanine ammonia-lyase (PAL), and pathogenesis-related genes (PR1 and PR-P2), in leaves, fruits (both green and red), and suspension-cultured cells of Micro-Tom. Effects of short exposure to UV-C, but not to UV-A, on induction of cell death (in cell suspension) and development of lesions accompanied by ion leakage (in the leaves) were observed while no morphological change was observed in the UV-treated green and red fruits. UV-dependent induction of PR genes (PR1 and PR-P2) in these samples suggested that UVs can be used for plant defense activation. In addition, expression of ACS1a was shown to be negatively and positively regulated by UV-C and UV-A, respectively. Thus UV-dependent post-harvest controls of fruit maturity and shelf-life are likely applicable (*i.e.* retardation and/or acceleration of maturation).

1. Introduction

Plants are naturally exposed to ultraviolet (UV) rays mostly at UV-A range (wave length, 320-400 nm), especially on sunny days in summer. In contrast, the level of harmful UV-C (wave length >220 nm) and UV-B (wave length, 280-320 nm) reaching the ground surface which potentially damages living organisms including plants could be effectively blocked and minimized by the presence of ozone layer above the stratosphere (Staehelin *et al*., 2001). However, especially in the southern hemisphere, the area of seasonal depletion of the ozone layer often expands outside the Antarctic region, occasionally allowing temporal increases in solar UV-B and relatively long-wave range of UV-C reaching the Earth's surface (Staehelin *et al*., 2001). Therefore, from the global point of view, studies on the plant-damaging impact of UV rays have key importance to both biologists and environmental researchers.

In general, irradiation with a high dose UV-C results in induction of programmed cell death in living plants. In seedlings and protoplasts of *Arabidopsis thaliana*, a dose of UV-C around 10-50 kJ m^{-2} induces an oligonucleosomal DNA fragmentation which is reminiscent of the apoptotic DNA laddering often described in mammalian cells (Danon and Gallois, 1998). In addition, UV-C-dependent cell death development in *Arabidopsis* involves caspase-like activity which is also similar to the events in animal apoptosis (Danon *et al.*, 2004).

On the other hand, a pulse or low dose of UV-C radiation is frequently used for direct removal of germs from fresh produce (Stevens *et al*., 1998). For instance, exposure of fruit tissue slices of zucchini squash to low dose UV-C

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reportedly lowers the microbial activity and prevents deterioration of fruit quality during subsequent storage at low temperatures, while a burst in respiration rate in the treated tissues is induced (Erkan *et al*., 2001). In addition to direct removal of pathogenic microbes from the plant surface by UV, researchers have reported attempts to use UV radiation for so-called 'plant hormesis' by which the susceptibility of plants to pathogens could be minimized and shelf-lives of fresh produce could be extended (Stevens *et al*., 1996, 1998). To date, various fruits and vegetables including sweet potatoes (Stevens *et al*., 1990, 1999), apples (Lu *et al*., 2007), peaches (Stevens *et al*., 1998; Lu *et al*., 2007), and tomatoes (Liu *et al*., 1993) have been tested for UV-mediated disease resistance controls. In most of the cases, resistance against pathogens was induced by UV-C (Stevens *et al*., 1999).

It is likely that treatment with UV rays (including UV-C) builds up the "immunity" against microbial infection in living plants, thus the UV hormesis effects described above may be, at least partially, attributed to stimulation of plants' innate immunity. The innate immune system of plants against pathogenic microbes is known to be elicited in response to recognition of pathogen-derived molecules by plant cells, as reviewed elsewhere (Dangl and Jones, 2001; Chisholm *et al*., 2006; Yoshioka *et al*., 2008). Recognition of such elicitors by the host cells' transmembrane receptors (Jones and Dangl, 2006; Altenbach and Robatzek, 2007) or resistance (R) proteins (Allen *et al*., 2004; Chisholm *et al*., 2006; Dodds *et al*., 2006) reportedly initiates the cellular signaling cascades which finally activate the defense mechanisms.

Micro-Tom, a dwarf cultivar of tomato (*Solanum lycopersicum* L.), originally produced for ornamental purposes, has been proposed as preferred plant material for molecular biological research, mainly because of its compact habitat and short life cycle (Meissner *et al*., 1997; Eyal and Levy, 2002; Marti *et al*., 2006). Recently, AbuQamar *et al*. (2009) suggested that Micro-Tom is a good model for studying the crosstalk between biotic and abiotic stress responses through regulation of gene expression.

In the present study, cellular damage (*viz*. cell death increase in suspension culture and increase in ion leakage at tissue level) and preceding changes in the gene expression patterns, especially those of defence-related, redox-related, DNA maintenance-related and fruit maturation-related genes, are shown to be induced by UV-A and UV-C in the leaves, fruits and suspension-cultured cells of Micro-Tom. The signalling mechanisms contributing to the regulation of UV responses are also discussed by analogy to plant immunity responses. Furthermore, the discussion addresses UV-dependent extension of the storage life of tomato fruits through stimulation of plant immunity mechanism by post-harvest exposure to UVs.

2. Materials and Methods

Plant materials

Following the protocols described elsewhere (Kadono *et al*., 2009), seeds of tomato (*Solanum lycopersicum* L., *cv*. Micro-Tom) were allowed to germinate on a wet paper towel in a transparent plastic container placed in a light-cycle-conditioned incubator (12 h light and 12 h dark at 23°C). Immediatly after germination the resulting plantlets were re-planted in plastic pots filled with a standard soil mixture and watered daily in the light-cycle-controlled incubator for three months. From the adult plants, leaflets, green immature and red ripe fruits (minimum size, 15 mm in diameter) were harvested before and after irradiation with UV lights.

Preparation of cell suspension culture

Again, following protocols described elsewhere (Kadono *et al*., 2009), cell suspension culture derived from Micro-Tom was prepared. Briefly, the leaf slices were taken from a seedling of Micro-Tom grown *in vitro* and placed on a MS agar plate containing 2,4-dichlorophenoxyacetic acid (0.2 μg ml^{-1}) to promote the formation of calli. Suspension culture of cells was initiated by addition of the sliced calli into the MS liquid medium (pH 5.8). The cells suspended in 30 ml of media in 100 ml-conical flasks were kept on gyratory shakers (at 130 rpm) at 23°C in darkness, with occasional sub-cultures by innoculating the fresh media with 3 ml of confluent culture. After about six months of continuous propagation of the cells with constant sub-culturing (initially twice a month and later once a week), a stable cell line was obtained.

For experimental purposes, the culture was pre-conditioned as follows. The confluent culture was used to inoculate the fresh MS liquid medium (3 ml culture to 30 ml medium) and pre-cultured for three days. Then 6.5 ml of each pre-culture was transferred to 100 ml of fresh MS liquid medium (in a 500 ml conical flask) and further cultured for three days. The resultant three-day-old large scale culture (at log-phase) was harvested and used for the experiments.

UV treatments

Intact and/or excised leaves (leaflets and leaf disks), green and red fruits, and suspension-cultured cells of Micro-Tom were irradiated with UV-C (254 nm) or UV-A (365 nm) using a handy UV trans-illuminator (SLUV-4, As-one, Osaka, Japan). The intensities of UV-A and UV-C were monitored with UV meters (UVX-25, UVP Inc., Upland, CA; YK-34UV, Lutoron Electronic Enterprise Co., Ltd., Taipei, Taiwan) and the intensity of UV-A and UV-C applied to the surfaces of plant materials were adjusted to 2.2 mW cm^{-2}, therefore the doses of UV irradiation were controlled by altering the length of irradiation time. Leaflets and fruits were exposed to UV rays immediately after harvest. To prevent water loss from the leafy samples during UV irradiation and incubation, leaflets and leaf discs were floated on ultrapure water in Petri dishes. For treatment of the cultured cells, 1 ml of cell suspension was added to each well on 12-well microplates and used for irradiation with UV rays. Irradiation time varied as indicated in the Results section.

Measurement of ion leakage from leaf discs

Measurements of ion leakage were performed using the discs of leaflets floated on the ultrapure water. Leaf discs were irradiated with UV rays for 30 min and further in-

cubated in darkness for up to 24 h. Each single well on a 12-well microplate was filled with 5 ml of ultrapure water and used to float three leaf discs (diameter, 9 mm) freshly prepared from the leaflets. Using a handheld conductivity meter (CD502A, Custom, Tokyo, Japan), monitoring of the changes in conductivity in the bathing liquid (with 1 h intervals up to 10 h) were carried out following 24 h of post-UV incubation. An increase in conductivity reflects the leakage of ions from the UV-dependently damaged leaves. The extent of ion leakage was expressed as the ratio (percentage) of recorded conductivity to the maximal conductivity obtained after boiling the samples.

Evaluation of cell death

Analysis of induced cell death in suspension culture was carried out as described elsewhere (Iwase *et al*., 2014). Following UV-treatments, 200 µl-aliquots of cell suspension were sampled and transferred into 1.5 ml tubes and statically incubated in darkness (for 2 h unless indicated). Then, 0.1 % (w/v) Evans blue was added to the cell suspension and further incubated for an additional 1 h. Following repeated washes with fresh media, counting of the stained cells was performed under microscopes (SMZ800 and Labophoto, Nikon, Tokyo, Japan; VHX-100, Keyence, Tokyo, Japan) and the level of cell death was quantified. For statistical analysis, three to four different digital images of cells under the microscope (each covering 50-100 cells to be counted) were acquired and analyzed.

RNA isolation following UV irradiation

Isolation of RNA followed by reverse-trancriptase polymerase chain reaction (RT-PCR) was carried out basically as described (Kunihiro *et al*., 2011). Leaflets and fruits were subjected to irradiation with UV rays for 30 min, and were sampled and frozen in liquid N_2 at 0, 1 and 10 h after irradiation. Post-UV incubation was carried out in darkness.

Cell suspensions on 12-well microplates subjected to 15 min of irradiation with UV rays were further incubated for 1 h in the darkness. Cells were then harvested by filtering through 40-µm pore nylon mesh and washed with fresh liquid culture medium. The obtained samples were frozen in liquid N_2.

These frozen samples were ground using a pestle and mortar and transferred to plastic tubes and extracted with the RNA extraction buffer containing 100 mM Tris-HCl (pH 8), 100 mM ethylenediaminetetraacetic acid, 100 mM LiCl, and 1% sodium dodecyl sulfate. Then aliquots of 1:1 mixture of phenol and CIA (chloroform:isoamyl alcohol at 24:1) were added and samples were subjected to centrifugation at 14,000 *g* for 10 min at 4°C. The upper layer collected in separate tubes were further subjected to extraction with aliquots of phenol and CIA and centrifugation. The resultant upper layer was again collected in new tubes and mixed with 1/3 volume of 10 M LiCl and kept at -30°C for 2 h. Following centrifugation, the resultant pellets were collected and re-suspended in 2 M LiCl and spin-collected. The pellet formed was dissolved in TE buffer and added with 1/2 volume of both phenol and CIA. The samples were subjected to 10 min of centrifugation and the upper layer was collected and mixed with the aliquot of CIA, and further centrifuged. For precipitation of RNA, the upper layer was collected and mixed with 1/10 volume of 3 M sodium acetate and 2.5 volume of absolute ethanol, and centrifuged. The pellet washed with 70% ethanol was spin-collected, dried and dissolved in diethylpyrocarbonate-treated water.

RT-PCR

Prior to analysis with RT-PCR, genomic DNA concomitantly present in the total RNA preparations was eliminated with RNase-free Cloned DNase I (Takara Bio Inc., Otsu, Japan). First-strand cDNA synthesis was performed using SuperScript™ III reverse transcriptase (Invitrogen Corporation, CA). The reaction mixtures (20 µl/tube) contained total RNA (2 µg), oligo-$(dT)_{20}$ (2.5 mM), and dNTP mixture (2 mM). The tubes were heated to 65°C for 5 min, cooled and kept at 4°C for 1 min using Program Temp Control System PC-320 (ASTEC, Fukuoka, Japan). To the reaction mixture, 4 µl of 5x First-Strand buffer, 1 µl of 0.1 M dithiothreitol, 1 µl of RNaseOUT recombinant RNase inhibitor and 1 µl of SuperScript™ III reverse transcriptase (200 units μl^{-1}) were added and incubated at 50°C for 60 min. The reaction was terminated by heating at 70°C for 15 min and cooling at 4°C for 15 min. The resultant cDNA solution was used for PCR performed with 60 ng of first-strand cDNA and Takara ExTaq™ (Takara Bio Inc., Otsu, Japan). For each sample, 30 cycles of PCR were performed with denaturing at 94°C for 1 min, annealing for 1 min and elongation at 72°C for 1 min. Sequences of the primers used and anealing temperatures employed are listed in Table 1.

Table 1 - List of primers used for RT-PCR

Genes studied	Accession numbers *	Forward primers	Reverse primers	Tm (°C)
Actin	U60481	cacactgtccctatttacga	gtaataacttgtccatcagg	51.3
ACS1a	U72389	gctttgggttagtttcagctc	gttcatgaactcatgatccaatc	56.5
APX	DQ096286	gagtacctcaaggctgttgacaaatg	gagcctcagcaragtcagcaaag	60.0
PR-P2	X58548	ggagagagttaacaagttgtgtg	gagtagtattaaaagttagctcg	60.0
PR1	DQ159948	cttctcatggtattagcc	ccaccatccgttgttgc	50.3
PAL	M83314	gacacacaagttgaagcatcac	cacatcttggttgtgttgctc	56.5

* Accession numbers were of NCBI GenBank.
Tm= melting temperature used for annealing.

3. Results

Cellular damage

On the UV-C-treated leaves, the damaging impact of UV-C at cellular level could be visualized as spotted lesions appeared (Fig. 1). Such visible damage was not observed on the UV-A-treated leaves. In addition to visible symptoms, UV-C irradiation resulted in a marked increase in ion leakage from leaf disc preparations (Fig. 2). Leakage of ions indicates that the cellular membrane is one of the targets of the damaging impact of UV-C irradiation.

After irradiations with UV rays at 2.2 mW cm^{-2}, suspension-cultured cells of Micro-Tom showed development of cell death depending on the type of UVs, exposure time and the length of post-exposure incubation (Fig. 3). Compared to UV-A, impact of UV-C irradiation was shown to be much more severe, requiring shorter irradiation time and post-irradiation incubation.

UV-responsive gene expressions

The genes examined were 1-amincocyclopropane-1-carboxylic acid (ACC) synthase gene (ACS1a), ascorbate peroxidise gene (APX, cytosolic isoform), pathogenesis-related (PR) genes PR1 and PR-P2, and phenylalanine ammonia-lyase gene (PAL) as possible targets of UV impact, and *actin* gene as non-inducible reference.

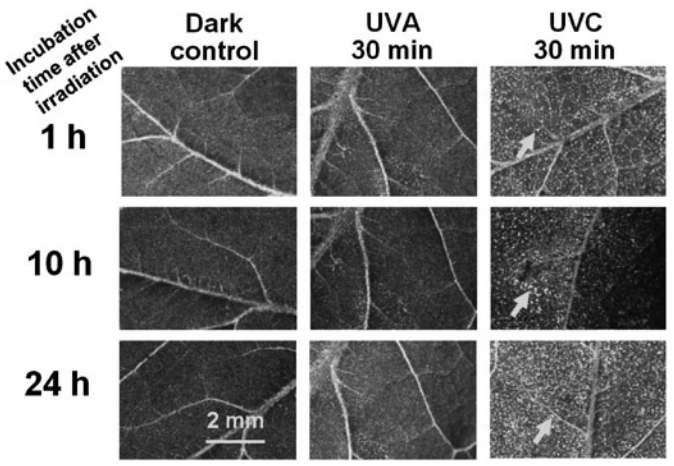

Fig. 1 - Development of UV-induced symptoms (lesions) on the leaves of Micro-Tom after the UV-irradiation. Leaflets were irradiated with UV rays for 30 min and further incubated in darkness on ultrapure water. Lesions on the leaves were observed under microscopes. Typical images from three repeated experiments are shown.

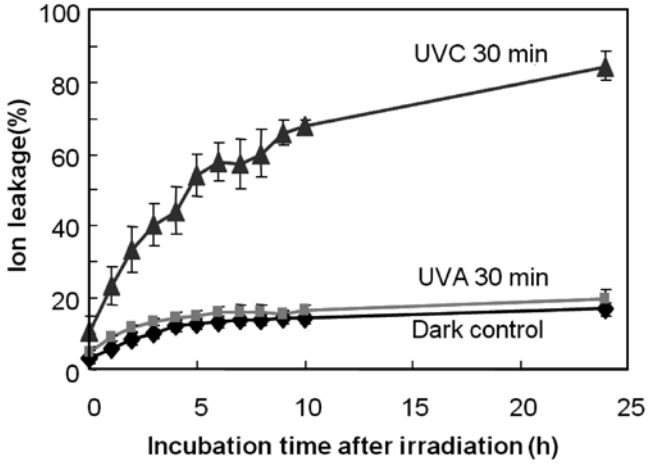

Fig. 2 - Measurement of ion leakage from the UV-irradiated leaf discs of Micro-Tom. Leaf discs were irradiated with UV rays for 30 min and further incubated in darkness on ultrapure water. The percentage of ion leakage was calculated as a ratio to conductivity after boiling of the leaf samples. Each data point and error bar reflect the mean and S.D., respectively (n = 3).

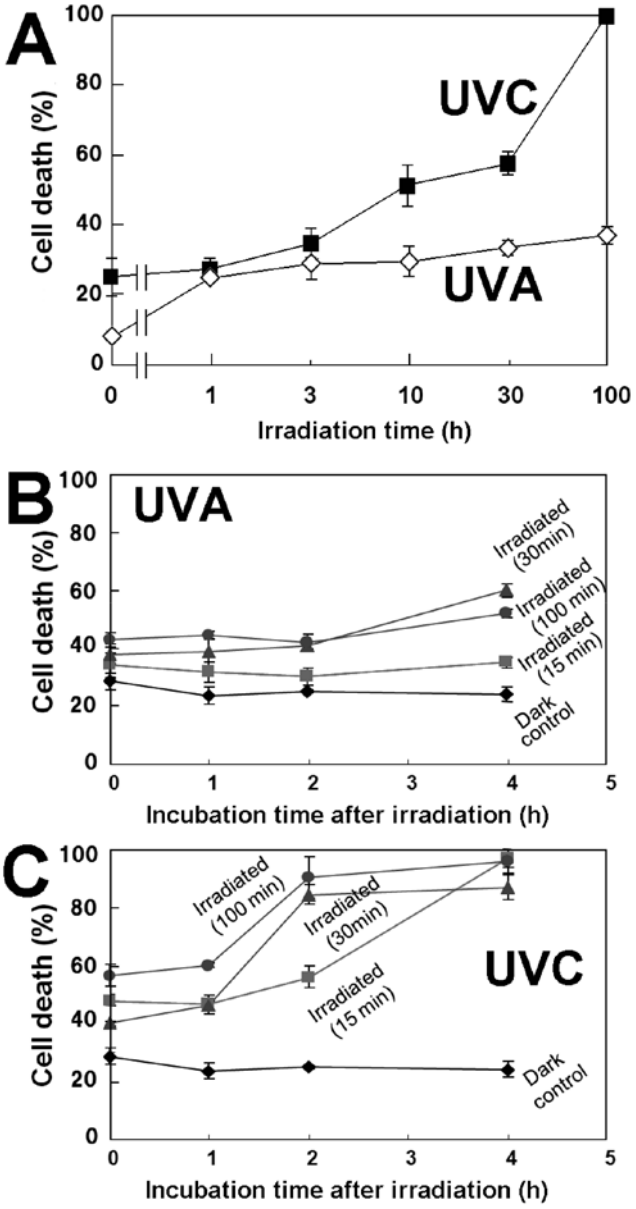

Fig. 3 - UV-induced cell death in suspension cultured cells. (A) Effect of irradiation time on the UV-induced cell death. (B) Progress of cell death after the UV-A-irradiation. (C) Progress of cell death after the UV-C-irradiation. Cell death was judged by Evans blue staining under microscopes. Each data point and error bar reflect the mean and S.D., respectively (n = 3).

In the cell suspension culture of Micro-Tom, expression of APX, PR-P2, PAL, and actin were shown to be maintained at active level even prior to the irradiation with UV rays (Fig. 4 A, dark control). In the leaf samples of Micro-Tom, ACS1a, APX, PAL, PR-1 and actin were shown to be expressed without UV irradiations (Fig. 4 B). Similarly, mature red fruit samples revealed high expressions of ACS1a, APX, PR-1 and *actin*, prior to the irradiation with UV rays (Fig. 5 B). In contrast, in the green fruit samples, only APX and actin were expressed in the dark control (Fig. 5 A).

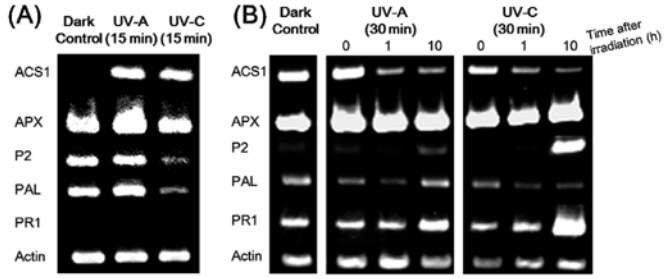

Fig. 4 - RT-PCR analysis of UV-responsive gene expressions in cell suspension and green leaves. Typical RT-PCR profiles of gene expressions in UV-irradiated cell suspension (A) and leaf samples (B) are shown. *Actin* was used as an internal control. Note: comparison of the density of bands on several gels were performed after gathering the images of gels on the black background.

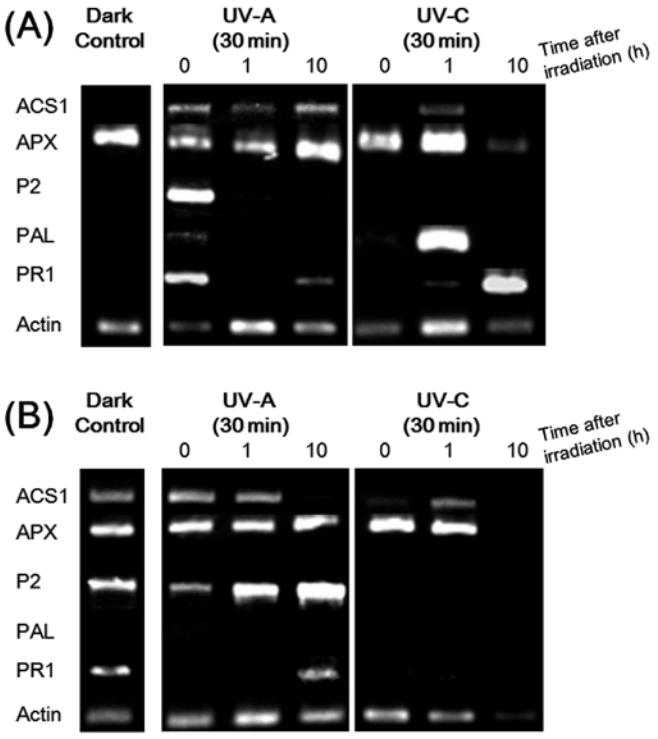

Fig. 5 - RT-PCR analysis of UV-responsive gene expressions in green fruits and red ripe fruits. Typical RT-PCR profiles of gene expressions in UV-irradiated green fruits (A) and red ripe fruits (B) are shown. *Actin* was used as an internal control. Note: comparison of the density of bands on several gels were performed after gathering the images of gels on the black background.

Among the genes tested, ACS1a was the only gene UV-dependently activated in the cell suspension culture. Expression of ACS1a was induced by both UV-A and UV-C (Fig. 4 A). UV-A-dependent activations of PR1 in the green fruits and leaves, of ACS1a in the green fruits and cell suspension culture, and of PAL and PR-P2 in the green fruits were observed (Fig. 4 and 5). UV-C-dependent activation of PR1 in green fruits and leaves, of PR-P2 in the leaves, of PAL in green fruit tissue, and of ACS1a in the green fruits and also in the cell suspension culture were observed (Fig. 4 and 5). Although PR1 was shown to be responsive to both UV-A and UV-C in green fruit tissue, the temporal profiles of induced gene expression largely differed. In green fruits, UV-A and UV-C were shown to be rapid and slow inducers of PR1 expression, respectively (Fig 5).

Since, most of the genes examined were active in the dark control of red fruit samples, drastic activation of gene expression could not be observed, except for the case of enhancement of PR-P2 expression which was originally active in the dark control.

APX was shown to be active in all materials even prior to UV irradiation, thus only the suppressive impacts of UV irradiations could be expected with this gene. Suppression of *APX* expression was observed in the UV-C-treated red fruit samples at 10 h after irradiation. Similarly, following irradiation with UV-C, expressions of PR-P2 and PAL, which were constitutively active in the cell suspension, were eventually suppressed. Expression of ACS1a in the leaves and red fruits was shown to be suppressed by UV-A irradiation. Expression of ACS1a, PR-P2, and PR1, originally active in red fruits, were shown to be suppressed by UV-C treatments.

4. Discussion and Conclusions

Cell death or defense activation?

A number of researchers have documented toxic impacts of UV rays in living plants including the damages to DNA, inhibition of photosynthesis, generation of reactive oxygen species (ROS) (Roldán-Arjona and Ariza, 2009). On the other hand, there have been some attempts to develop the UV irradiation protocol for so-called 'plant hormesis' by which the susceptibility of plants to invading pathogen is minimized and thus shelf-life of fresh produces is likely extended (Lu *et al*., 2007). For example, Kunz *et al*. (2008) demonstrated that UV-C-induced DNA damage in *Arabidopsis* accompanies the dose- and time-dependent development of resistance against *Hyaloperonospora parasitica*. Interestingly, it has been shown that, even in the absence of UV-C, plant nucleotide excision repair mutants displayed the identical type of resistance to *H. parasitica*, suggesting a positive role for UV-C-mediated DNA damaging events in plant immunity activation.

APX, one of the typical antioxidant genes, encodes for the enzyme capable of H_2O_2 elimination, thus protecting

the plants from the damaging impacts of ROS. PR genes (*viz.*, PR-P2 and PR1) and PAL are related to the actions and production of salicylic acid (SA). SA is a hormone-like natural signaling molecule involved in the defense response against infection by pathogens in higher plants. SA acts by stimulating the production of PR proteins through a complex signaling mechanism involving ROS, calcium and protein phosphorylation in the early stages (Kawano *et al*., 1998; Kawano and Bouteau, 2013). In *Solanaceae* plants including tobacco and tomato, the increase in *PAL* expression reportedly results in accumulation of SA (Kawano *et al*., 2004).

Here, the short exposure to UV-C but not to UV-A resulted in an acute increase in cell death in the cell suspension culture (Fig. 3). Development of lesions (visible symptoms), (Fig. 1) accompanied by ion leakage (sign of membrane damage) (Fig. 2) was induced in the UV-C-treated leaf samples. In contrast, no visible damage was observed in the UV-treated green and red fruits. It is noteworthy that UV-A or sub-lethal low dose UV-C was shown to induce the defense-related genes (*PR1* and *PR-P2* genes) in the leaves (Fig. 4 B) and green fruit tissues (Fig. 5 A).

The above data imply that cell death (possibly apoptotic, data not shown) and plant protection mechanisms (expression of antioxidant genes and PR genes) are induced by high and low doses of UV-C, respectively. Therefore, moderate doses of UV irradiation should be applicable to plants to confer tolerance to a variety of abiotic stresses (chiefly, oxidative stress) and innate plant immunity (represented by the induced expression of PR genes).

Possible involvement of SA signaling

Our data are comparable to the work of Marco *et al*. (2008) who reported the ozone-induced gene expression controls in the leaves of three tomato cultivars (*viz.*, Nikita, Alisa Craig and Valenciano). Similar to our data on UV-treated Micro-Tom, expression of *APX* is reportedly suppressed in the ozone-treated Nikita tomato (Marco *et al*., 2008). In addition, the tomato leaves chronically exposed to ozone showed activation of redox-related and defense-related genes such as *PAL* (Marco *et al*., 2008). Thus, ozone-induced gene expression patterns and the UV-induced gene expression patterns may share a common regulatory mechanism.

One of the key pathways that may be common to ozone response and UV response in tomato is the SA signaling pathway. Expressions of PR genes and *EDS1*, known factors functioning upstream of SA-dependent expression of PR genes (Falk *et al*., 1999), were induced by ozone in three tomato cultivars (Marco *et al*., 2008). In the present investigation, activation of *PAL* and PR genes in the UV-treated Micro-Tom leaves (Fig. 4B) was observed, suggesting the possible involvement of a SA signaling path.

Recently, in the culture of *Arabidopsis* cells, we observed that over-expression of bacterial salicylate hydroxylase gene (NahG) and pharmacological reagents targeting either ROS or calcium signaling effectively blocked both the cell death induction by high-dose UV-C and induction of defense-related gene expressions by sub-lethal low-dose UV-C (Hiramatsu *et al*., unpublished results). Requirements for early calcium signaling, proven by aequorin luminescence and the action of calcium targeted pharmacological reagents, and generation of ROS, are commonly observed in various plant models during the responses to SA (Kawano *et al*., 1998; Kawano and Muto, 2000; Kawano and Bouteau, 2013), pathogen-derived molecules (Kadota *et al*., 2004), ozone (Kadono *et al*., 2006, 2010; Tran *et al*., 2013), peroxyacetyl nitrate (Yukihiro *et al*., 2012), toxic metal ions (Lin *et al*., 2005; Kagenishi *et al*., 2011; Kunihiro *et al*., 2011), and UV (Hiramatsu *et al*., unpublished results).

Possible regulation of SA action by UV-C

According to the work by Nawrath *et al*. (2002) using *Arabidopsis thaliana*, expression of EDS5, a member of multidrug and toxin extrusion transporter family, known to be involved in the accumulation of SA and *PR1* transcript, is very low in unstressed plants but strongly induced by attacks by pathogens, treatment with SA, and irradiation with UV-C. EDS5 expression induced by pathogen infection and UV-C exposure largely depends on the pathogen response proteins EDS1, PAD4, and NDR1, suggesting the requirements for the signal transduction pathways commonly employed in UV-C responses and defence responses (Nawrath *et al*., 2002). This defense mechanism-dependently induced *EDS5* transcript reportedly starts accumulating 2 h after exposure to UV-C, and the transcription level likely remains for two days, thus further contributing to the long-lasting SA responses. This view shall be studied in detail in our future experiments.

Possible role of chlorophylls.

Our data indicate that the response in green samples (leaves and immature fruits) and non-green samples (ripe fruits and suspension cultured cells) might differ in their modes of responses to UVs. While PR1 expression, one typical measure of stress responses, was induced by UV-C in green tissues, non-green samples (both the ripe fruits and cell suspensions) showed no induction of PR1 expression (Fig. 4 and 5). It is tempting to speculate that the chlorophyll-related compounds may behave as secondary active signals for mediating the stress responses. One of such candidate compounds may be pheophorbide *a* derived from chlorophyll *a*, which is known to be produced in the ethylene-exposed green tomato fruits (Kawano *et al*., 1999). Recently, it was revealed that pheophorbide *a* plays a key role in both light-dependent and light-independent cell death mechanism in plants (Hirashima *et al*., 2009). This aspect is worth testing in future experiments.

Regulation of ethylene production

ACS1a coding for ACC synthase is a ripening-related gene responsible for production of ethylene precursor, ACC. Since ethylene promotes the ripening of fruits and senescence of leaves, UV-dependent changes in ACS1a expression may drastically affect the life-cycle of the

plants and shelf-life of the fruits. This ethylene biosynthesis-related gene was shown to be activated by both UV-A and UV-C in the cell suspension culture and green fruits, suggesting that UV treatments may be available for post-harvest enhancement of fruit maturity. Interestingly, UV-A and UV-C irradiation to red fruits resulted in gradual lowering of the ACS1a expression level (Fig. 5), suggesting that UVs are possibly applicable for retardation and prevention of ethylene-mediated fruit over-ripening and softening.

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Analytical statistical interpretation of relationship between different parameters of kiwifruit (*Actinidia deliciosa* cv. Hayward) during cold storage

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Key words: ascorbic acid, correlation, kiwifruit, total antioxidant activity, total phenolic compounds.

Abstract: Physicochemical and metabolic changes of kiwifruit characteristics were studied during storage. Parameters, such as total phenolic compounds (TPC), color (*L, hue, chroma), total antioxidant activity (TAA) and ascorbic acid (AA) content, were evaluated at harvest and every 15 days during fruit storage at 0°C and 90% ± 5 RH. Correlations between different parameters were also evaluated. The results of this study suggest that high antioxidant capacity in kiwifruit is due to a strong association (r^2>0.90) between AA and TPC, and that phenolic compounds and AA are the major contributors to the antioxidative activities of *Actinidia deliciosa* cv. Hayward. Color parameters were found to have weak correlation coefficients with TPC, TAA and AA. It is worth noting that the physical-chemical parameters do not have a relationship with the chromatic parameters.**

1. Introduction

Kiwifruit (*Actinidia deliciosa*) is cultivated mainly due to its sensory properties and its ability for prolonged cold storage. This latter attribute enables consumption of kiwifruit throughout the year in many parts of the world (Minas *et al*., 2010). The most widely grown *Actinidia* cultivar is the *A. deliciosa* cv. Hayward. Commercial production of this variety has spread to many countries because of its distinctive characteristics, including fruit size, high productivity, and sufficient storageability (Ferguson, 1999).

Kiwifruit is a good source of natural antioxidant substances, in particular vitamin C (Nishiyama *et al*., 2004). In fact, the content of vitamin C in kiwifruit ranges between 25 and 155 mg/100 g of fresh weight (FW) of fruit (Tavarini *et al*., 2009), making it higher than that determined in orange, strawberry, lemon and grapefruit. Beever and Hopkirk (1990) showed that vitamin C content in kiwifruit was tenfold higher than the same content found in apple and peach. Esti *et al*. (1998) have observed that the vitamin C content of kiwifruit depends on genotype, ripening degree, storage and the analysis method utilized. Kiwifruit's strong antioxidant capacity is due to a wide number of phytonutrients including carotenoids, lutein, phenolics, flavonoids and chlorophyll (Kaya *et al*., 2008). In fact, during fruit ripening, several biochemical, physiological and structural modifications occur and these changes determine the final fruit quality attributes (Lee and Kader, 2000; Ayala-Zavala *et al*., 2004).

During postharvest storage of horticultural crops, important changes in antioxidant status can occur (Ayala-Zavala *et al*., 2004). Kiwifruit is rich in bioactive compounds, especially polyphenols (Park *et al*., 2009). Polyphenolic compounds are a complex group of substances that have gained enormous attention in recent years, especially within the analytical chemistry field because they exhibit important quality properties and antioxidant activity (Escarpa and González, 2001). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994).

The dark green color of kiwifruit, due to the chlorophylls in plastids in the pericarp cells of the flesh (Talens *et al*., 2002), can be modified during storage. Most fleshy fruits are green only during the earlier stages of development: they undergo dramatic changes in chemical composition and ultra-structure during maturation and ripening. Associated with tissue softening and the changes in carbohydrate and organic acid metabolism there is conversion of chloroplasts into chromoplasts and a concomitant loss of chlorophyll, often accompanied by accumulation of carotenoids (Montefiori *et al.*, 2009). The pigments responsible for the flesh color in ripe fruit of *A. deliciosa* have already been described (McGhie and Ainge, 2002; Nishiyama *et*

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al., 2005); the color is mainly due to chlorophylls a and b (Nishiyama *et al*., 2005).

The changes in bioactive compounds of kiwifruit that occur during cold storage have not previously been studied, thus in this paper we describe changes in pericarp color, ascorbic acid (AA), total antioxidant activity (TAA) and total phenolic contents (TPC) in the fruits with different storage times. The differences and correlations between these parameters are also examined.

2. Materials and Methods

Plant material

Mature, unripe kiwifruit (*Actinidia deliciosa* cv. Hayward) of medium size (80-120 g), free from visible defects or decay, were harvested from a commercial kiwifruit orchard in Gorgan, Iran with average firmness of 10 (kg/cm^2) and 7% °Brix. Fruits were immediately transferred to the postharvest laboratory at Shiraz University. Kiwifruits were individually labeled and packaged into ventilated bags, then stored for four months at 0±1°C and 90±5% relative humidity (RH). Samples were taken at monthly intervals during storage for quality evaluation and analyses.

Physical and physicochemical assays

*Total phenolic contents.*The total phenolic contents of each extract were determined according to the method of Gutfinger (1981). Extracts (1 ml) at 1 mg/ml concentration were mixed with 1 ml of 2% Na_2CO_3. After standing for 3 min, 0.2 ml of 50% Folin-Ciocalteu reagent was added to the mixture and left to stand for 30 min. The mixture was centrifuged at 13 400×*g* for 5 min. The absorbance was measured at 750 nm and TPC are expressed as gallic acid quivalents (GAE).

Color. Color was determined using digital imaging (Afshari-Jouybari and Farahnaky, 2011). Fruit was photographed in a chamber; angle light with the horizontal surface of the images was 45 degrees. After transferring the images to a computer, Photoshop image processing software was performed. Individual *L**, *a** and *b** parameters were recorded: *L** is lightness, *a** [-greenness to +redness) and *b** (-blueness to + yellowness) are chromacity coordinates. The *a** and *b** values were converted to chroma (C* = $(a^{*2} + b^{*2})^{1/2}$) and *hue* angle ($h° = \tan^{-1}(b^*/a^*)$].

DPPH radical scavenging activity (RSA). The free radical scavenging activity was measured by 2,2-diphenyl-2- picrylhydrazil (DPPH) on the basis of the method of Brand-Williams *et al*. (1995) with minor modifications. For this determination, aliquots (0.1 mL) of the extract were mixed with 1 mL of a DPPH solution (500 µM) in 80% ethanol. The mixture was incubated at room temperature for 30 min. Solution absorbance was determined at λ =515 nm. The DPPH radical concentration was calculated using the following equation: scavenging effect (TAA %) = (1 - Af/Ao) × 100; Ao stands for the absorbance of the control sample and Af for the absorbance in the presence of the sample. L-ascorbic acid was used for the calibration curve, and the results are expressed as mg L-ascorbic acid equivalent. 100 g^{-1} fw (fresh weight).

Ascorbic acid. Ascorbic acid was measured by the oxidation of ascorbic acid with 2, 6-dichlorophenol endophenol and the results are expressed as mg/100 g fresh weight (Rangana, 1977).

Statistical analysis. Four replicates of each sample were used for statistical analysis. Correlation analyses between different parameters were carried out using the correlation and regression programme in MINITAB 16. Correlations were obtained by Pearson's correlation coefficient (r) in bivariate linear correlations. All statistical analyses and correlations were carried out with SAS software package v. 9.1 for Windows. Differences at P<0.05 were considered to be significant.

3. Results and Discussions

Ascorbic acid

Ascorbic acid (AA) content first increased up to 30 days (47.74 mg/100 g^{-1}) and then decreased (Table 1) and could be a result of its synthesis during the initial storage period. The observed variation (increase) of AA content is due to fruit weight loss by dehydration (hence due to a higher concentration) or actually to AA *ex novo* synthesis. Utilization of AA during later storage periods may be the reason for its decreased amounts. The accumulation of AA during ripening depends on the type of fruit. Lee and Kader (2000) reported that AA content increased with ripening in apricot, peach and papaya, but decreased in apple and mango. Generally, when fruits become overripe, vitamin C

Table 1 - Changes in AA, TPC, TAA and chromacity of kiwifruit during storage

Storage time (day)	Ascorbic acid (mg/100 g FW)	Total phenolic compounds (mg/100 g DW)	Total antioxidant activity (mg/ 100 g FW)	Color parameters		
				L*	Hue	Chroma
0	40.17±1.53 b	45.75 ±0.99 c	489.17±42.01 bc	51.9 ± 0.83 a	66.92±1.01 a	48.42±1.75 a
30	47.74 ±1.38 a	57.9±1.91 a	602.17±43.97 a	46.07±0.55 b	64.63±2.95 ab	45.49±1.19 b
60	40.97±2.17 b	50.65±3.35 b	556.20±70.33 ba	41.34±0.78 c	62.20±0.73 bc	39.61±0.77 c
90	35.68±2.11 c	43.3±2.56 c	434.34±47.60 dc	35.35±1.50 d	61.82±1.33 bc	35.68±1.16 d
120	27.44±1.89 d	30.45±1.65 d	354.18±40.84 d	39.92±0.82 c	59.76±1.41 c	32.62±1.73 e

Means within each column with different superscript letters are significantly different (p < 0.05) for each sampling.

content declines concurrently with the degradation of fruit tissues (Kalt, 2006).

In persimmon fruits, AA and TPC content showed a linear relationship with a positive correlation coefficient of r^2=0.976 (Fig. 1). Other authors found strong correlations between AA and TPC in different fruits (Gonçalves *et al*., 2004; Serrano *et al*., 2005). Ascorbic acid content was positively correlated with antioxidant activity (r^2 = 0.944), suggesting that AA makes a significant contribution to the total antioxidant capacity of kiwifruit (Fig. 2).

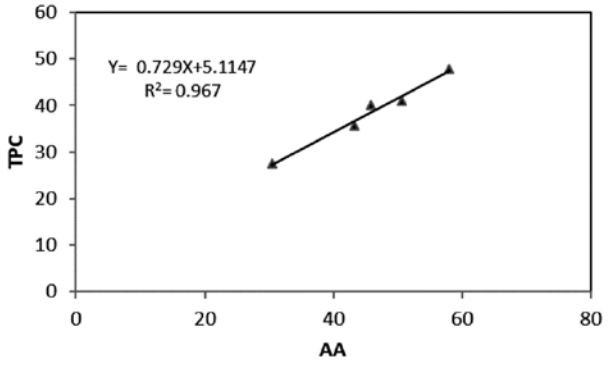

Fig. 1 - Correlation between TAA and AA in kiwifruit.

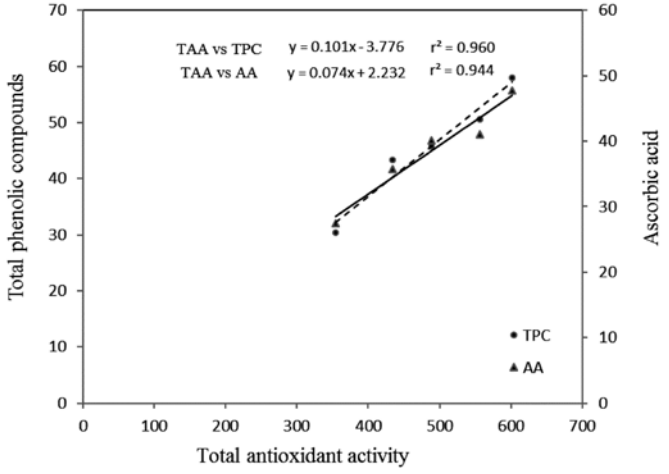

Fig. 2 - Correlation between TAA and total phenolic compounds or ascorbic acid in kiwifruit.

Total phenolic compounds

Total phenolic compounds of fruits increased up to 30 days (57.9 mg/100 g^{-1}) and then decreased (Table 1). During storage, TPC increased initially, probably due to synthesis from sugars, and decreased later due to its participation or utilization in other metabolic processes. Tavarini *et al*. (2008) reported that TPC may increase or decrease in fruits and vegetables, depending on the storage conditions. The increase in TPC during storage may be the result of fruit damage and tissue disruption during storage. Phenolic compound synthesis in response to wounding has been reported (Saltveit, 2000).

All o-quinone molecules are highly reactive and may interact with other phenols or other substances, co-polymerise and thus produce compounds, which determine undesired fruit browning, or oxidise further compounds, reduce to original phenols, or react with different nucleophile compounds such as amines, thiols, imidazole, and indole. Generally there is a positive correlation between TPC availability and vulnerability to PPO attack (Ramírez *et al*., 2003). Phenolic compounds represent the main substrates used by oxidative enzymes, having consequences in terms of color and quality changes, as well as being associated with plant defense mechanisms against stress situations that can affect the postharvest period (Tomás-Barberán and Espín, 2001).

Good correlation between TPC and AA was observed with a high significance level (P<0.001), and a similar relationship (0.97) was also obtained between TPC and TAA (Table 2). This positive and significant relationship between TPC and TAA was greater compared to AA and TAA. The results indicate strong association between antioxidative activities and phenolic compounds, suggesting that the latter are probably responsible for the antioxidative activities of kiwifruit. Phenolic compounds are also effective hydrogen donors, making them good antioxidants (Rice-Evans *et al*., 1995). Reports in literature on the relationship between TPC and TAA are contradictory; some authors have observed a high correlation (Proteggente *et al*., 2002; Tsao *et al*., 2003; Khanizadeh *et al*., 2008).

Table 2 - Correlation matrix (Pearson correlation coefficients)

	TPC	AA	TAA	Chroma	Hue	L*
TPC	1.00					
AA	0.98***	1.00				
TAA	0.97**	0.97**	1.00			
Chroma	NS	NS	NS	1.00		
Hue	NS	NS	NS	0.97**	1.00	
L*	NS	NS	NS	0.89*	0.86*	1.00

NS= not significant.
* Significant to 0.05 *p* level.
** Significant to 0.01 *p* level.
*** Significant to 0.001 *p* level.

Total antioxidant activity

TAA increased and peaked over the course of one month (57.9 mg/100 g^{-1}) then decreased toward the end of the storage period (Table 1). There is debate in the literature about the influence of vitamin C on the antioxidant capacity of fruits and vegetables (Guo *et al*., 2003). However, it is also known that fruits with high antioxidant capacity generally contain more antioxidants and most of these antioxidants have been shown to be phenolic compounds, in particular flavonoids (Guo *et al*., 2003). For example in pomegranate, ascorbic acid and phenolic compounds are responsible for the TAA, alone or in combination (Kulkarni and Aradhya, 2005). Examining the entire storage period, it can be observed that changes in antioxidant activity

were very similar to phenolic compounds (Lemoine *et al*., 2009). TAA was highly and positively related to AA (r^2 = 0.944) (Fig. 2). However, in the present study, the best correlation ($r^2 > 0.96$) was observed between TPC and RSA (Fig. 2). This fact probably indicates that the antioxidant capacity of kiwifruits is primarily due to TPC and AA.

Color

A significant decrease in hue angle was observed during the storage of fruits at 0°C, indicating continued ripening during cold storage. The initial L* value was 51.9. Internal lightness decreased sharply within 90 days of storage and then increased significantly until the end of storage (Table 1). The chroma (*C**) value was initially 48.42 (Table 1). Chroma values (internal) of fruits decreased during the entire storage period and reached their minimum values at the end of storage. Changes in *C** result principally from a loss of chlorophyll content, mostly chlorophyll *a* which decreases during storage (Fuke *et al*., 1985). In kiwifruit, lightness and chroma values significantly decreased during cold storage and shelf-life, indicating less color intensity (Koukounaras and Sfakiotakis, 2007). When fruits darken, skin color becomes less chromatic and surface browning develops. Diminished red skin and darkening due to oxidative browning reactions have been found to be more marked in ripe strawberries that suffer greater moisture loss during storage (Nunes *et al*., 2005).

Correlation analyses showed that in the fruit tissues, most of the correlation coefficients were lower, positive and not significant. However, the correlation between chroma of kiwifruit and TPC, TAA and AA was weakly positive and significant (Table 2). These results indicate that the physical-chemical parameters do not have a relationship with the chromatic parameters and TAA, TPC and AA in kiwifruit. Similar results were reported by Drogoudi *et al*. (2008) and Vieira *et al*. (2009).

4. Conclusions

Taken together, the results of this study imply that high antioxidant capacity in kiwifruit is due to a strong association between AA and TPC; color parameters of kiwifruit were found to have high correlation coefficients with TPC. It is worth emphasizing that these latter have been correlated with the degree of browning as a result of decreased antioxidative activities.

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Biorational insecticides against the potato tuber moth (Lepidoptera: Gelechiidae) on stored potatoes

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Key words: larvicidal, ovicidal, *Phthorimaea operculella*, potato storage, reduced-risk insecticides.

Abstract: This study was conducted to evaluate the residual activity and efficacy of spinosad, emamectin benzoate, and chromafenozide on potato tuber moth, *Phthorimaea operculella*. Almost 0% egg hatch of 1-1.5 and 4-4.5-day-old eggs occurred when eggs were treated topically with spinosad at a concentration of 216 mg/L. No ovicidal activity was observed when emamectin benzoate and chromafenozide were tested against the eggs at concentrations of 5, 10, 15 and 37.5, 75 mg/L. Spinosad and emamectin benzoate were equally highly toxic to larvae (100% mortality) even when they were used at low rates. A relatively small proportion of F_1 adults (≈11 to 20%) emerged in the chromafenozide treatment at concentrations of 37.5 and 75 mg/L. One hundred percent larval mortality was noted when potato tubers were sprayed with spinosad and emamectin benzoate and stored for at least 90 days after application. Whereas, chromafenozide applied at 75 mg/L was effective in reducing moth emergence, exhibiting activity for 14 days only after application; thereafter a similar number of F_1 adults occurred in chromafenozide and control treatments. Thus, spinosad and emamectin benzoate could be used to efficiently protect potato tubers from *P. operculella* infestation for three months in unrefrigerated rustic potato stores.

1. Introduction

The potato tuber moth, *Phthorimaea operculella* (Zeller) is a widely distributed oligophagous pest of solanaceous crops, including potato, tomato, tobacco, and other cultivated or uncultivated Solanaceae (Cameron *et al*., 2002). Due to the economically important damage it causes, this pest has a long history of exposure to a broad array of synthetic insecticides. Not surprisingly, *P. operculella* has developed resistance to many of these insecticides, including chlorinated hydrocarbons, organophosphates, carbamates, and pyrethroids (Dillard *et al*., 1993; Symington, 2003).

In the past decade, several classes of conventional insecticides have emerged that show great promise for controlling *P. operculella* (Edomwande *et al*., 2000; Saour, 2008; Clough *et al*., 2010). Spinosad is a naturally-derived biorational insecticide with a relatively benign toxicology profile (Aydin and Gürkan, 2005). It is comprised primarily of two macrocyclic lactones, spinosyn A and D, secondary metabolites produced by the actinomycete, *Saccharopolyspora spinosa*, under natural fermentation conditions (Thompson *et al*., 2000). Insects ingesting spinosad experience paralysis caused by rapid excitation of the nervous system through binding to the nicotinic acetylcholine and/or gamma-aminobutyric acid (GABA) receptors (Salgado, 1998). Currently, spinosad is registered in over 60 countries and is used to control Lepidoptera pests in many vegetables, fruits, and field crops (Legocki *et al*., 2010; Wang *et al*., 2013).

Emamectin benzoate (an epi-methyl amino derivative of abamectin) is a second-generation avermectin analog with exceptional activity against lepidopterous on a variety of vegetable crops worldwide (Ioriatti *et al*., 2009). Avermectins (a 16-membered family) are naturally occurring macrocyclic lactones isolated from fermentation products of the soil micro-organism *Streptomyces avermitilis* (Ishaaya *et al*., 2002). Emamectin benzoate causes irreversible activation of chloride channels in the nervous system of insects. Shortly after contact or feeding exposure, the insect larvae stop feeding, become irreversibly paralyzed and die in three to four days. Due to its rapid photodegradation by sunlight, contact activity of emamectin benzoate against insect predators or parasites is limited to a very short period, allowing selective control of some Lepidoptera pests (Sechser *et al*., 2003).

Chromafenozide is a relatively novel insecticide against lepidopteran larvae characterized by a methylchromane moiety in its dibenzoylhydrazine structure. Its mechanism of action is similar to nonsteroidal ecdysone agonists known as an insect-specific ecdysis hormone (Nakagawa, 2005). Although this compound is very toxic to insects, it is safe for mammals and is environmentally benign (European Food Safety Authority, 2013). Chromafenozide on plants is ingested orally by insect larvae to exhibit an ecdysis-promoting activity and lead to death. It is effective in controlling various lepidopterous pests

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(i.e. Tortricidae, Pyralidae, Noctuidae) on vegetables or other agricultural plants (Yanagi *et al*., 2006).

In reviewing the literature, no experimental data were found related to the direct contact or residual activity of these insecticides against *P. operculella* under storage conditions. Thus, the current study was performed to determine the potential effects of three insecticides - spinosad, emamectin benzoate, and chromafenozide - on the embryonic and early larval stages of the potato tuber moth. Moreover, this study was designed to evaluate the residual activity of the insecticides tested at various intervals up to 90 days after application.

2. Materials and Methods

Insects

Insects used in the experiments were obtained from a laboratory stock culture, which is supplemented each year with field-collected *P. operculella* larvae (infested tubers). Larvae were reared on wax-coated potato slices placed in plastic containers (40x25x10 cm). The newly emerged moths were collected and confined in 800 ml transparent plastic jars (10-12 pairs in each jar). A band of filter paper was added to the bottom of each jar for oviposition and 10% sucrose solution was presented as a food source. The rearing procedures were conducted at a constant temperature of 25±1°C with 70±5% relative humidity (RH) and a photoperiod of 12:12 h (L:D).

Insecticides

The commercially formulated insecticides used in the present study were spinosad (Spintor® 2 SC, 240 mg [AI]/ml), emamectin benzoate (Proclaim® 05 SG, 50 g [AI]/kg), and chromafenozide (Matric® 5% SC, 50 g [AI]/l).

Ovicidal effect

After the copulation and oviposition period, the first batch of eggs deposited on the oviposition support (white filter paper) from each pair were removed, counted and approximately divided into two groups. The eggs were counted with the aid of a binocular stereo microscope (Kyowa Optical SDZ-PL, Japan). The first group was used for insecticide treatments and the second served as a control. Eggs were kept in a small transparent plastic box (4x3x2 cm) and held at a constant temperature of 25±1°C with 70±5% RH, until used.

Treatments were prepared by diluting each commercial formulation of the aforementioned insecticides in 1 L water. *P. operculella* eggs aged 1-1.5 and 4-4.5 days deposited on the oviposition support were dipped for 30 s in three different concentrations 72, 144, 216 ml/L and 5, 10, 15 mg/L for spinosad and emamectin benzoate, respectively (0.3, 0.6, 0.9 ml/L of Spintor and 0.1, 0.2 and 0.3 g/L of Proclaim); while chromafenozide was applied at two concentrations of 37.5 and 75 mg/L (0.75 and 1.5 ml/L of Matric). The concentrations used did not exceed the manufacturer's recommended application rates. A non-ionic organic surfactant (Agral® 600 g/L nonyl phenol ethylene oxide condensate) was added at 0.15 ml/L as a wetting agent to ensure good dispersal of the preparation. After treatments, the eggs were air-dried and held for incubation. A 0.15 ml/L surfactant solution was used as a control in the experiments. Seven days following treatment the percentage of eggs hatch was recorded. The experiment consisted of three replicates for each concentration of each insecticide with a set of 1000 eggs per replicate for each age group.

Larvicidal effect

Experiment 1. Healthy, medium-sized potato tubers of 130-150 g weight (n≈150) were sprayed to runoff with spinosad, emamectin benzoate, and chromafenozide including surfactant at the above-mentioned concentrations, while the untreated tubers (control) were misted with surfactant solution. All tubers were then allowed to air dry at room temperature. After treatments, tubers were deposited over a layer of sand in transparent plastic containers (20x12x10 cm, three tubers per container). First-instar of *P. operculella* larvae (<16 h old) were gently placed on the treated and untreated potato tubers (three larvae per tuber) using a fine camel-hair brush and held at the rearing conditions described above. The number of emerged F_1 adults was recorded. The experiment was conducted three times using a total of 1080 larvae (45x3 larvae for each concentration of each insecticide).

Experiment 2. The experiment was carried out to determine whether or not larvae hatched from insecticide-treated eggs are able to survive if they are allowed to develop on untreated tubers (from a practical point of view this is only possible when insecticide-treated and untreated potato heaps were stored together). As described above, 1-1.5- day-old eggs on the filter paper bands were dipped for 30 s in the respective insecticide preparations, air-dried, cut to small sections containing ten eggs and fixed on the surface of uninfested potato tubers using mini paper pins (one egg section per tuber). Eggs of the control were treated with surfactant-added water solution (0.15 ml/L). The tubers were placed in transparent plastic containers (three tubers per container) with a layer of sand completely covering the bottom of the container and held at the rearing conditions. The tubers were visually inspected and the number of emerged F_1 adults was noted. The experiment was repeated three times for each concentration of each insecticide with 120 eggs per replicate.

Residual activity

Four heaps (≈50 kg each) of healthy, medium-sized potato tubers were thoroughly sprayed with the respective insecticide preparations at the highest concentrations 216, 15 and 75 mg/l for spinosad, emamectin benzoate, and chromafenozide, respectively. Water/surfactant solution was used for the control treatment. After drying, the tubers were stored in the dark at room temperature (≈23°C) in order to reduce tuber weight loss and the accumulation of solanine (Haddadin *et al*., 2001; Gachango *et al*., 2008).

Newly hatched larvae were placed on the tubers at 0, 7, 14, 21, 28, 35, 42, 49, 60 or 90 days after insecticide applications (three larvae per tuber and three tubers were placed in each plastic container). The number of emerged F_1 adults was recorded. Three replicates were retained for each stored period of each insecticide with 45 larvae per replicate.

Statistical analysis

Differences in egg hatchability and emergence of F_1 adults were tested by the analysis of variance (ANOVA) at the 5% level (P<0.05). Significant ANOVAs were followed by the protected least significant difference (PLSD) at $\alpha < 0.05$. Differences in egg hatch between 1-1.5 and 4-4.5-day-old eggs were determined using paired-samples *t*-test (StatView Version 4.02; Abacus Concepts, 1994). Data were arcsine transformed prior to analysis to stabilize the variance. Dose-mortality responses were estimated by probit analysis (IBM, 2010). Percentages of egg hatching and adult emergence were corrected according to Schneider-Orelli's (Kroschel and Koch, 1996) formula:

$$\% M = (b - k/100 - k) \times 100$$

where M = corrected %, b = % observed in the treatment, and k = % observed in the control.

3. Results

Among the insecticides tested, ovicidal activity was observed only in spinosad preparations (Table 1). Spinosad at the median and high concentrations (144 and 216 mg/l) was very effective in controlling *P. operculella* egg hatch compared to emamectin benzoate, chromafenozide and control treatments. There were no significant differences in egg hatchability between emamectin benzoate and chromafenozide used at 5 and 37.5 mg/L and the control for 1-1.5-days-old eggs (F= 476.2; df= 8, 261; P< 0.0001). The LC_{99s} for 1-1.5-day-old eggs were 240.81, 97.17 and 530.32 mg/L for spinosad, emamectin benzoate and chromafenozide, respectively. 1-1.5-day-old eggs were more sensitive to emamectin benzoate and chromafenozide than 4-4.5-day-old eggs (t= 6.3; df= 29; P<0.0001 for emamectin benzoate used at 15 mg/L concentration).

A drastic reduction in the percentage of F_1 emerged adults was observed when insecticide-treated tubers were offered to *P. operculella* neonate larvae (Table 2). Adult emergence was completely inhibited in spinosad and emamectin benzoate treatments compared with 72.2% in the control (F= 474.2; df= 8, 126; P<0.0001). However, 20.3 and 11.3% of F_1 adults successfully emerged in chromafenozide treatment at 37.5 and 75 mg/L, respectively.

Significant differences were observed in the mean percentage of F_1 adults emerged from larvae that hatched from insecticide-treated eggs and fed on untreated potatoes compared to the control (F= 1616.5; df= 8, 99; P<0.0001) (Table 3). However, no F_1 adults emerged with spinosad treatment at 0.6 and 0.9 mg/L, while 13.4 and 18.3% of adult emergence was noted in emamectin benzoate and chromafenozide at 0.1, and 37.5 mg/L, respectively.

The time-dependent efficacy of the tested insecticides against *P. operculella* is presented in Table 4. The residual activity of spinosad and emamectin benzoate used at 216 and 15 mg/L remained unchanged (zero F_1 adults

Table 1 - Mean (±SE) hatchability of potato tuber moth eggs of two age groups treated topically with spinosad, emamectin benzoate, and chromafenozide insecticides

Insecticides	Chemical group	Active ingredient mg/l	% of hatched eggs [z]	
			1-1.5 day old egg	4-4.5 days old egg [y]
Control	-		91.9±4.2Aa	94.1±3.9Aa
Spinosad	Spinosyns	72	12.7±3.9Ac	15.8±3.8Ac
(Spintor® 2 SC, 240 mg/ml)		144	0.0±0.0Ac	6.0±1.5Bd
		216	0.0±0.0Ac	1.6±0.4Ad
Emamectin benzoate	Avermectins	5	86.8±5.3aBb	93.7±3.3Aa
(Proclaim® 05 SG, 50g/kg)		10	78.6±4.0Bab	87.4±4.5Aab
		15	70.3±5.1Bb	86.3±5.1Ab
Chromafenozide	Non-steroidal	37,5	84.6±5.0Bab	91.6±4.1Aab
(Matric® 5% SC, 50g/l)	ecdysteroid agonist	75	76.9±3.9Bb	84.7±4.8Ab

[z] Means in row for each egg age followed by the same uppercase letter are not significantly different (P < 0.05, *t*-test); means in column for each egg age followed by the same lowercase letter are not significantly different (P < 0.05, Fisher PLSD).

[y] Data were corrected according to Schneider-Orelli's formula and arcsine transformed prior to analysis; mean of three replicates, 1000 eggs per replicate for each insecticide and concentration.

Table 2 - Mean percentage (±SE) of potato tuber moth F_1 adults emerged from spinosad-, emamectin benzoate-, and chromafenozide-treated and untreated potato tubers

Insecticides	Chemical group	Active ingredient mg/l	F_1 emerged adults (%) [z]
Control			72.2±7.84 a
Spinosad (Spintor® 2 SC, 240 mg/ml)	Spinosyns	72	0.0±0.0 d
		144	
		216	
Emamectin benzoate (Proclaim® 05 SG, 50 g/kg)	Avermectins	5	0.0±0.0 d
		10	
		15	
Chromafenozide (Matric® 5% SC, 50 g/l)	Non-steroidal	37,5	20.3±2.1 b
	ecdysteroid agonist	75	11.3±2.8 c

[z] Means in column followed by the same letter are not significantly different (P<0.05, Fisher PLSD); data were corrected according to Schneider-Orelli's formula and arcsine transformed prior to analysis; mean of three replicates, 45 larvae per replicate for each concentration of each insecticide.

Table 3 - Mean percentage (±SE) of potato tuber moth F_1 adults emerged from spinosad-, emamectin benzoate-, and chromafenozide-treated and untreated eggs, the hatched larvae being fed on untreated tubers

Insecticides	Chemical group	Active ingredient mg/l	F_1 emerged adults (%) [z]
Control			68.9±8.4a
Spinosad	Spinosyns	72	7.1±3.1d
(Spintor® 2 SC, 240 mg/ml)		144	0.0±0.0e
		216	
Emamectin benzoate	Avermectins	5	13.4±1.6c
(Proclaim® 05 SG, 50g/kg)		10	8.1±2.0d
		15	0.0±0.0e
Chromafenozide	Non-steroidal	37,5	18.3±4.6b
(Matric® 5% SC, 50g/l)	ecdysteroid agonist	75	13.1±5.1c

[z] The treated eggs were at the 1-1.5 day-old egg stage; means in column followed by the same letter are not significantly different (P<0.05, Fisher PLSD); data were corrected according to Schneider-Orelli's formula and arcsine transformed prior to analysis; mean of three replicates, 120 eggs per replicate for each concentration of each insecticide.

Table 4 - Mean percentage (±SE) of potato tuber moth F_1 adults emerged from spinosad-, emamectin benzoate-, and chromafenozide-treated and untreated potato tubers several days after treatment

Stored period, days after insecticides application	F_1 emerged adults (%) [z]			Control
	Spinosad (216 mg/ml)	Emamectin benzoate (15 mg/l)	Chromafenozide (75 mg/l)	
7	0.0 ± 0.0 c	0.0 ± 0.0 c	11.7 ± 1.5 b	70.3 ± 8.9 a
14	0.0 ± 0.0 c	0.0 ± 0.0 c	23.4 ± 4.1 b	72.1 ± 9.9 a
21			62.2 ± 6.9 a	68.9 ± 7.2 a
28			65.1 ± 6.1 a	67.9 ± 7.8 a
35			68.0 ± 9.9 a	71.0 ± 9.9 a
42			65.7 ±7.8 a	66.9 ± 6.9 a
49			68.2 ± 9.8 a	69.1 ± 9.0 a
60			70.0 ± 9.9 a	71.4 ± 9.7 a
90	0.0 ± 0.0 b	0.0 ± 0.0 b	66.3 ± 7.5 a	68.0 ± 9.6 a

[z] Means in row followed by the same letter are not significantly different (P<0.05, Fisher PLSD data were corrected according to Schneider-Orelli's formula and arcsine transformed prior to analysis; mean of three replicates, 45 larvae per replicate for each stored period of each insecticide.

emergence) for up to 90 days after application. In contrast, a severe loss in efficacy occurred at the end of the two-week test period for tubers treated with chromafenozide. Significant differences were obtained when chromafenozide was compared to the other insecticides tested (F= 5714.3; df= 3, 56; P<0.0001 after 14 days of storage), indicating that spinosad and emamectin benzoate were more efficient in protecting potato tubers from *P. operculella* infestation than chromafenozide even during 14 days after application.

4. Discussion and Conclusions

In general, there are few studies regarding the effect of insecticides on eggs of Lepidoptera pests and most publications focus on the control of the larval stage, consequently, little information is available about the effect of insecticides on eggs of *P. operculella*. Regardless of egg developmental stages, nearly 100% mortality (or 0% egg hatch) was obtained when eggs were treated with spinosad at high and medium concentrations (216 and 144 mg/L, respectively), which was not the case for emamectin benzoate and chromafenozide. Therefore, it is obvious that spinosad has excellent embryocidal activity and the compound was able to penetrate the chorion (eggshell) and reach the developing embryos. Our results concerning the efficacy of spinosad on *P. operculella* egg hatchability agree with several authors who found an excellent ovicidal activity of spinosad when applied against the eggs of the Egyptian cottonworm *Spodoptera littoralis* (Boisd.), the cactus moth *Cactoblastis cactorum* (Berg), the cranberry fruitworm, *Acrobasis vaccinii* Riley and the diamondback moth *Plutella xylostella* (L.) (Bloem *et al*., 2005; Temerak, 2005; Wise *et al*., 2010; Mahmoudvand *et al*., 2011). On the other hand, El-Barkey *et al*. (2009) reported that a 52% reduction in percentage of egg hatching was obtained when eggs of the pink bollworm *Pectinophora gossypiella* (Saunders) were challenged with Radiant Sc 12% (the second generation of spinosad) at the LC_{50} level. However, according to Perez *et al*. (2007), spinosad showed no ovicidal properties when applied against eggs of the *Aedes aegypti* (L.) mosquito.

When *P. operculella* eggs were topically treated with emamectin benzoate and chromafenozide either at low and medium, or at high concentrations, egg hatchability was not prevented. However, at the highest concentrations, a weak ovicidal effect was noted and eggs hatching were significantly reduced by 8.3 and 10% at the concentrations of 15 and 75 mg/L for emamectin benzoate and chromafenozide, respectively. Our data corroborate the results that emamectin benzoate used in field experiments at the rate of 13.5 g AI/ha had no ovicidal effect on the minute pirate bug *Orius albidipennis* (Reuter) (Sechser *et al*., 2003). In fact, emamectin benzoate was registered as lepidopteran larvicidal insecticide, which means that the product must be ingested by Lepidoptera larvae to be effective. Accordingly, Scarpellini (2001) reported that all larval stages of the cotton leafworm *Alabama argillacea* Hübner died 12 h after they started eating cotton leaves treated with emamectin at 9.6 g AI/ha.

Not surprisingly, we found no effect of chromafenozide on the mean percentage of egg hatch. Chromafenozide, like other inhibitors of ecdysteroid biosynthesis, shows toxic effects against larvae of lepidopteran pests mainly via digestion (Yanagi *et al*., 2006). In this respect, Slama (1995) reported that the ecdysone agonists were completely ineffective in all methods of topical application against the ligated larvae of *Galleria mellonella* L., *Manduca sexta* (L.) and *Pieris brassicae* (L.). Nonetheless, Kandil *et al*. (2012) reported that the hatchability of 1-d-old eggs of *P. gossypiella* was 53% when they were treated with chromafenozide at the LC_{50} level compared with 97.0% in the control.

Our data show that the older eggs (4-4.5 days old) were relatively more tolerant to insecticides than the younger ones (1-1.5 days old). In general, the hatchability of Lepidoptera eggs following insecticide treatments depends on the compound, dose, and age of eggs (Gelbic *et al*., 2011). The relative sensitivity of newly laid eggs to insecticides compared to those of older age classes can be attributed, at least in part, to chorion hardening during the embryo development that obstructs the penetration of external products (Tavares *et al*., 2011).

P. operculella neonate larvae exhibited a high response (100% mortality) to all concentrations tested of spinosad and emamectin benzoate, however low numbers of larvae that challenged chromafenozide-treated tubers achieved their development and emerged as F_1 adults (the percentage of emerged adults was relatively high when the compound was used at the low concentration). These findings agree with the data reported by other researchers regarding the larvicidal efficacy of spinosad, emamectin benzoate and chromafenozide in controlling several lepidopteran pests (Kandil *et al*., 2012; Abouelghar *et al*., 2013; Dong *et al*., 2013; Nasir *et al*., 2013; Tong *et al*., 2013).

When *P. operculella* larvae hatched from spinosad-, emamectin benzoate-, and chromafenozide-treated eggs were fed on untreated tubers, the percentages of F_1 emerged adults were completely inhibited for spinosad and emamectin benzoate used at medium and high concentrations and drastically reduced for chromafenozide treatment. It seems probable that treated-egg chorion retained toxic residues to cause the death of newly hatched L1 larvae which puncture the eggshell and eat their way through while hatching. It's worth mentioning that when we evaluated the results concerning the emamectin benzoate and chromafenozide treatments, we found that *P. operculella* neonate larvae had been successful at creating a small opening in the eggshell during the process of exiting the chorion; however, most of the larvae failed to survive and died without feeding in close proximity to the egg. This indicates that although these two compounds do not have ovicidal activity, they have demonstrated ovi-larvicidal activity when applied topically after eggs have been laid. Our data concerning the ovi-larvicidal activity

of emamectin benzoate and chromafenozide are congruent with studies on the effects of these insecticides and other insecticides with ecdysone mode of action on *Helicoverpa armigera* Hübner and *A. vaccinii* (Dhadialla *et al*., 2005; Wise *et al*., 2010; Singh and Kumar, 2012).

Determination of residual activity of an insecticide is essential information to protect agricultural products from re-infestation. Spinosad and emamectin benzoate provide, under protected environments (complete darkness), a 100% residual control effect up to 90 days after application, while chromafenozide applied at 75 mg/L showed limited residual activity of 7 to 14 days. Therefore, *P. operculella* larvae exposed to chromafenozide-treated tubers at 21 days following treatment continued their development and reached the adult stage. The extended period of residual activity of spinosad and emamectin benzoate could be related to the rate of their photodegradation, since these two compounds are known to undergo relatively rapid photodegradation via photolysis (primary route of degradation) that ultimately affects their residual toxicity (Liu *et al*., 1999; Jones *et al*., 2005; Zhu *et al*., 2011). Our insecticide-treated potatoes were stored in total darkness and this could explain the slow degradation of spinosad and emamectin benzoate under our experimental laboratory conditions. On the other hand, Ditya *et al*. (2012) found that the dissipation rate (half-life) of chromafenozide applied to different types of soil samples in laboratory conditions was between 15.8 and 23.9 days.

In conclusion, the results of this study are the first published data on the efficacy of spinosad, emamectin benzoate and to a lesser extent chromafenozide against *P. operculella* eggs and neonate larvae and demonstrate that these compounds could be used as replacements for earlier insecticide classes. Moreover, spinosad and emamectin benzoate proved to be highly effective in protecting potatoes from *P. operculella* infestation almost completely for three months and therefore they could be successfully used in unrefrigerated rustic potato stores.

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Growth Studies in Mangosteen (*Garcinia mangostana L.*). II. Activation of seedling growth in Mangosteen using Arbuscular Mycorrhizal Fungi and *Azospirillum*

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Key words: Arbuscular mycorrhizal fungi, *Azospirillum, Garcinia mangostana, Glomus fasciculatum, Glomus mosseae.*

Abstract: An experiment was undertaken in the central orchard at the main campus of the Kerala Agricultural University to address the slow growth in mangosteen, a highly potential crop of the humid tropics. *Glomus mosseae, Glomus fasciculatum* and *Azospirillum* individually and in combinations, as well as a control, formed the treatments. The treatments were adequately replicated in a completely randomized design. The best treatments for activating seedling growth were the combinations of *Glomus fasciculatum* (5 g) + *Azospirillum* (10 g) + single super phosphate (10 g) followed by *Glomus fasciculatum* (5 g) + *Azospirillum* (20 g) + single super phosphate(10 g) per plant. A rhythmic pattern was observed with the treatments giving the best seedling growth also yielding higher values of nitrogen, phosphorus, potassium, crude protein, chlorophyll a, b, total chlorophyll, total phenol total carbohydrates and abscisic acid content; treatments with intermediate growth recording also gave intermediate values except in the case of sodium. Control plants gave values that fell beween those of intermediate and the least growth. The highest spore count was observed in plants inoculated with *Glomus fasciculatum* (20 g) + single super phosphate (10 g) followed by *Glomus fasciculatum* (20 g) + *Azospirillum* (20 g) + single super phosphate (10 g). With regard to root infection, plants inoculated with *Glomus fasciculatum* (5 g) + *Azospirillum* (20 g) + single super phosphate (10 g) per plant and *Glomus fasciculatum* (20 g) + *Azospirillum* (10 g) + single super phosphate (10 g) revealed the maximum percentage of infection. The *Azospirillum* population was highest in the plants inoculated with *Glomus fasciculatum* (5 g) + *Azospirillum* (10 g) + followed by *Glomus mosseae* (20 g) + *Azospirillum* (20 g) + single super phosphate (10 g). The standard procedure for identification and quantification of abscisic acid was modified, as clear banding patterns were not obtained. Using the modified procedure, the characteristic-banding pattern corresponding to standard abscisic acid was obtained and confirmed when standards of abscisic acid were also simultaneously used with samples. Banding patterns and quantification of samples of the treatment with *Arbuscuar mycorrhizal fungi* and *Azospirillum*-inoculated plants were also successfully obtained and are presented. Growth measurements at the end of the first year revealed that all characters recorded were far superior to the established selection indices for the purpose.

1. Introduction

Mangosteen (*Garcinia mangostana L.*), the "Queen of fruits" is a successful introduction into Kerala and flourishes well under the warm humid tropics (Yusuf and Kurien, 2012). It has high export potential but is limited by its long gestation period (Wiebel *et al.,* 1992, 1995). However, this long period of 10-15 years (Lim, 1984; Richards, 1990; Wiebel *et al.,* 1995) can be reduced by resorting to vegetative propagations; the problem of slow growth only gets magnified and the consequent low canopy volume leads to lower yield. Hence, mangosteen related work that will lead to activation of growth should get top priority in research.

The knowledge of symbiotic associations of mycorrhizal fungi with roots of vascular plants is a century old (Mohandas, 1993). Root infections by arbuscular mycorrhizal (AM) fungi have been reported in many perennial fruits such as grapes citrus, and apple. Root inoculated perennials with AM fungi effectively enhances the growth of plants such as plant height, number of leaves and leaf area. Inoculation of AM fungi in mangosteen resulted in significant changes in length-related characteristics (Masri *et al.,*

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1998). Other similar reports exist on increased growth in various crop plants including root growth enhancement as reported and reviewed by Gerdemann (1968) and Cherian (2001).

The role of AM fungi in increasing the mobilization and uptake of P and thereby the productivity of many crops is well documented and reviewed by Gerdemann (1968), Mosse, (1973), Meenakumari (1987), Nelsen (1987) and Bhandari *et al.* (1990). There are numerous reports of AM fungi increasing the N concentration in plant shoots and aiding in stimulating nodulation (Carling *et al.*, 1978). AM fungi directly enhances the uptake of micronutrients, viz. Zn, Cu and Fe (Gildon and Tinker, 1983; Kucey and Tanzen, 1987). Zinc deficiency can also be corrected by inoculating plants with an endomycorrhizal fungus (Gilmore, 1971). It was observed that AM fungi association resulted in a higher uptake of micronutrients in various plants, which was brought about by selective uptake and better utilization of N, Cu, Zn and S in various crops (Bhandari *et al.*, 1990)

With regard to *Azospirillum,* there is only scanty information available on its influence in perennial crops (Rao, 1982). Studies have been made in the rhizosphere and rhizoplane of cocoa and pepper (Govindan and Nair, 1984; Govindan and Chandy, 1985). However, most of the studies with *Azospirillum* are on field crops (Rao *et al.*, 1979). Rao and Dass (1989) found that soil inoculation with pure cell suspension of *Azospirillum brasilense* or *Azotobacter chrocaccum* resulted in growth enhancement of ber and pomegranate. Enhanced root elongation, root hair development and branching in a number of crops have been reported following *Azospirillum* inoculation (Kapulnik *et al.,* 1983). High crude protein content was noticed in inoculated plants (Patel *et al.*, 1993).

However, there are also reports on the dual inoculation of AM fungi and *Azospirillum* and its growth response in plants. Combined inoculation of *Azospirillum* and AM fungi significantly increased shoot biomass in mulberry (Nagarajan *et al*., 1989); plant height, shoot and root weight in pepper (Bopaiah and Khader, 1989); and shoot growth, and thereby biomass production, in *Tectonia grandis* (Sugavanam *et al*., 1998). Greater root colonization resulting in higher N and P and micro nutrients like Fe, Cu, Zn and Mn have been reported in coffee (Kumari and Balasubramanian, 1993). Sonowane and Konde (1997) revealed that co-inoculation of AM fungi and *Azospirillum* or *Azotobacter* resulted in the highest leaf area in studied grape vines and comparison between the two revealed that *Azospirillum* was superior when used in conjunction with a mixed culture of AM fungi (Sugavanam *et al*., 1998).

The probable reason for this increased dry weight was attributed to a higher photosynthetic rate (Estrada-Luna, 2000), or perhaps the production of growth promoting compounds namely auxin, gibberellins and cytokinins or vitamins (Miller, 1971; Crafts and Miller, 1974; Slankis, 1975).

Masri *et al.* (1998) observed that arbuscular mycorrhiza enhanced the growth and reduced the nursery period of mangosteen (*Garcinia mangostana* L.) seedlings. In mangosteen (*Garcinia mangostana* L.), alteration of root system characteristics and nutrient uptake in response to AM fungal inoculation have been studied. Arbuscular mycorrhizal inoculation induced significant changes in root characteristics and this was accompanied by a tremendous increase in nutrient uptake. Uptake of P was increased by 67-88% in inoculated seedlings (Masri and Azizah, 1998).

The present study was undertaken with the prime objective of ascertaining as to whether the growth rate in mangosteen can be increased through symbiotic association of AM fungi and Azospirillum. However, its presence and beneficial effects in cultivated crops of Kerala have been reported by various workers (Potty, 1978; Sivaprasad *et al*., 1982, 1984; Girija and Nair, 1985; Nair and Girija, 1986).

2. Materials and Methods

The experimental site experiences a warm humid tropical monsoon climate. It is situated at 12°32'N latitude and 74°20'E longitude at an altitude of 22.5 m above mean sea level. The study was carried out at the Kerala Agricultural University Central Orchard, Thrissur. The area receives an average rainfall of 2150 mm distributed over a year's period. The mean maximum temperature ranged 28-36°C and the mean minimum temperature 12.8-20.6°C. The relative humidity was 90-98% with a mean of 94%.

The soil type is typical sandy clay loam with a pH of 5.4, EC of 1.25 dsm^{-1} and belongs to the order Ultisols with 8 p^H ranging from 5.5-5.8. The soil is low in available N and $P_2 05$ and high in K_2O.

Fruits were collected from plants belonging to the same age group (25-50 years) from the Pariyaram area of the Thrissur District in Kerala and seeds were extracted. The seeds were sown in black polythene bags (45x30 cm) filled with potting mixture comprised of farmyard manure, sand and cow dung in the ratio 2:2:1. The weight of the potting media was 5 kg, which was uniformly maintained. Seedlings were subjected to a secondary selection for uniformity in growth. Three months after germination of the seeds, the treatments were inoculated in the potting media and a uniform dose of 10 g of single super phosphate was added to the potting media of each polybag. The treatments were as follows:

1. *Glomus mosseae* (G.m.) - 5 g
2. *Glomus mosseae* - 10 g
3. *Glomus mosseae* - 20 g
4. *Glomus fasciculatum* (G.f.) - 5 g
5. *Glomus fasciculatum* - 10 g
6. *Glomus fasciculatum* - 20 g
7. Azospirillum (Az.) - 5 g
8. Azospirillum - 10 g
9. Azospirillum - 20 g
10. Azospirillum - 5 g + *Glomus mosseae* - 5 g
11. Azospirillum - 10 g + *Glomus mosseae* - 5 g

12. Azospirillum - 20 g + *Glomus mosseae* - 5 g
13. Azospirillum - 5 g + *Glomus mosseae* - 10 g
14. Azospirillum - 10 g + *Glomus mosseae* - 10 g
15. Azospirillum - 20 g + *Glomus mosseae* - 10 g
16. Azospirillum - 5 g + Glomus *mosseae* - 20 g
17. Azospirillum - 10 g + *Glomus mosseae* - 20 g
18. Azospirillum - 20 g + *Glomus mosseae* - 20 g
19. Azospirillum - 5 g + *Glomus fasciculatum* - 5 g
20. Azospirillum - 10 g + *Glomus fasciculatum* - 5 g
21. Azospirillum - 20 g + *Glomus fasciculatum* - 5 g
22. Azospirillum - 5 g + *Glomus fasciculatum* - 10 g
23. Azospirillum - 10 g + *Glomus fasciculatum* - 10 g
24. Azospirillum - 20 g + *Glomus fasciculatum* - 10 g
25. Azospirillum - 5 g + *Glomus fasciculatum* - 20 g
26. Azospirillum - 10 g + *Glomus fasciculatum* - 20 g
27. Azospirillum - 20 g + *Glomus fasciculatum* - 20 g
28. Single Super Phosphate (SSP) - 10 g alone
29. Control

The morphological observations recorded were the plant height, girth at collar, total number of leaves, number of new flushes per year, number of leaves/flush and total leaf area and survival rate of the seedlings at one-year stage after germination stage. Leaf area was calculated by standardizing a common factor (0.6727) and then multiplying the length breadth with this factor (i.e. L×B×Factor). The leaf area was expressed as cm^2. The factor was pre-standardised taking 100 leaves and measuring the length and breadth. The leaf area of the corresponding leaf was measured using a leaf area meter to work out the factor value. Thus, the factor value (0.6727) was derived using the formula

Factor = (Leaf area/Length x breadth)

Using the factor value, the leaf area of a leaf/whole plant was calculated.

Survival rate was calculated by counting the established plants and expressing it as a percentage of the total number of seedlings observed after germination. Shoot and root fresh weight and dry weight, root to shoot dry weight ratio, length of longest root, number of primary, secondary and tertiary roots, and total number of roots were also recorded.

To estimate fresh weight of shoot and root, the seedlings were uprooted one year after germination. The plants were immediately cut and separated into shoots and roots. The fresh weights were recorded separately and the average expressed in grams. To obtain the dry weights, the samples collected to determine the fresh weights were dried in an oven maintained at 60°C till the weight of the samples remained constant. Dry weights were recorded separately and the average expressed in grams.

Dry weight ratio of root to shoot was calculated as follows:

Dry weight ratio = (Root dry weight/Shoot dry weight)

Length of the longest root (tap root) was measured from the collar region to the growing tip using a scale and expressed in centimeters.

For biochemical studies, leaf samples from seedlings were collected one year after germination; leaf samples from mother plants were also collected. The third leaf from the tip was collected and oven dried at 60°C, ground and used to estimate the content of N, P, K and Na. The following methods were applied, as described by Jackson (1973): for total nitrogen the microkjeldhal method was used and the average expressed as percentage; phosphorus content was determined using di-acid extract method; potassium content was determined with di-acid extract, then read in an EEL flame photometer, at 548 nm, and the average expressed in percentage; nitrogen content was estimated by microkjeldhal method; the value of nitrogen content was multiplied by the factor 6.25 to obtain the crude protein content and the average expressed in percentage; sodium content was determined with di-acid extract, then read in an EEL flame photometer at 598 nm and the average expressed in percentage.

The chlorophyll content (total chlorophyll, chlorophyll a and chlorophyll b) was estimated in leaf samples using Arnon's Acetone method (Sadasivam and Manickam, 1996) and the average expressed in milligrams.

The total sugars were estimated using standard procedure (AOAC, 1980), the total carbohydrates using Anthrone method (Dubois *et al*., 1951), and total phenol content using Folin-Ciocalteau method (Sadasivam and Manickam, 1996), all expressed in milligrams. The procedure adopted for quantification of abscisic acid was a modification of the standard method of Little *et al.* (1972). The modification became imperative as bands were not obtained. The procedure was standardized and bands were obtained corresponding to the standard abscisic acid. Further quantification was carried out using a U-V spectrophotometer and standards of known concentration from which a standard graph was obtained.

Microbial observations

Estimation of the spore population in the rhizosphere was carried out (Gerdemann and Nicolson, 1963) and expressed as number of spores/100/g soil.

Percent infection by AM fungi was calculated as described by Philips and Hayman (1970). The infection percentage was worked using the standard formula:

Percent infection= (Number of infected root segments/ Total number of root segments observed) x 100.

The *Azospirillum* population in the rhizosphere was estimated using serial dilution technique. The population was then calculated using an MPN table or chart (Cochran, 1950).

The study of all morphological and biochemical characters using a combination of arbuscular mycorrhizal fungi (AMF) + *Azospirillum* + single super phosphate was carried out as a completely randomized block design using Analysis of Variance techniques. Another set of plants receiving identical treatments were maintained for destructive analyses for taking root, shoot characters and biochemical analysis. The significance was tested by F test and the treatments were compared by Duncan's multiple range test (Snedecor and Cochran, 1983).

3. Results

Morphological characters of seedlings

All mean data on morphological characters of seedlings are presented in Table 1. At the twelve-months stage, maximum height was observed in the plants inoculated with G.f. (5 g)+ Az. (10 g)+ SSP (10 g), which was significantly superior to all other treatments except the next best combination of inoculation, G.f. (5 g) + Az. (20 g) + SSP (10 g). The greatest significant increment in height was recorded in plants inoculated with the same treatment as at the six-months stage, followed by plants treated with SSP (10 g) alone. These treatments were statistically superior in terms of rate of height increment compared to other treatments. Maximum girths at twelve months were recorded in the same treatment combinations as above, with both treatments statistically significant. Also at the twelve-months stage, the greatest increment was observed in the treatment combination G.f. (5 g) + Az. (20 g) + SSP (10 g), which was significantly superior to G.m. (10 g) + Az. (20 g) + SSP (10 g). These two treatments were significantly superior to all other treatments including the control.

With regard to the total number of leaves at the twelve-months stage, maximum leaf count was recorded with

Table 1 - Morphological characters of twelve-month-old mangosteen (*Garcinia mangostana* L.) seedlings inoculated with arbuscular mycorrhizal fungi (AMF) and *Azospirillum*

Treatments (g)	Twelve month stage (Nine months after inoculation)									
	Height (cm)	Increment in height (cm)	Girth (cm)	Increment in girth (cm)	Total number of leaves	Increment in total number of leaves	New flushes/ year (No)	Leaves/ flush (No)	Total leaf area (cm^2)	Survival rate (%)
G.m. 5	10.13 c	2.43 bcd	1.31 d	0.09 d	12.67 abcde	2.00 abcd	1.57 ab	2.00	82.61 bc	100.00 a
G.m. 10	7.73 c	1.33 d	1.62 abcd	0.11 cd	8.00 e	1.33 abcd	1.12 b	2.00	18.93 c	100.00 a
G.m. 20	11.23 bc	1.33 d	1.77 abcd	0.25 abcd	12.33 abcde	3.33 ab	1.67 ab	2.00	31.66 c	100.00 a
G.f. 5	8.93 c	1.27 d	1.38 cd	0.08 d	11.00 abcde	1.67 abcd	1.54 ab	2.00	42.43 bc	100.00 a
G.f. 10	11.57 bc	1.67 d	1.68 abcd	0.21 abcd	14.33 abcd	1.67 abcd	2.00 a	2.00	107.00 abc	100.00 a
G.f. 20	8.43 c	1.60 d	1.52 abcd	0.23 abcd	8.67 de	0.67 cd	1.22 ab	2.00	40.29 c	100.00 a
Az. 5	10.50 c	2.75 bcd	1.65 abcd	0.24 abcd	11.00 abcde	1.33 abcd	1.60 ab	2.00	111.10 abc	100.00 a
Az. 10	10.47 c	2.07 d	2.13 abcd	0.50 abcd	9.67 bcde	0.33 d	1.42 ab	2.00	84.49 bc	100.00 a
Az. 20	10.33 c	2.30 bcd	1.60 abcd	0.18 bcd	10.33 abcde	1.67 abcd	1.55 ab	2.00	47.77 bc	100.00 a
Az. 5 + G.m. 5	8.70 c	3.63 bcd	1.51 abcd	0.18 bcd	9.00 cde	1.00 bcd	1.21 ab	2.00	20.00 c	100.00 a
Az. 10 + G.m. 5	11.23 bc	3.10 bcd	1.76 abcd	0.30 abcd	13.00 abcde	2.00 abcd	1.80 ab	2.00	89.48 abc	100.00 a
Az. 20 + G.m. 5	8.83 c	2.70 bcd	1.54 abcd	0.20 abcd	11.33 abcde	2.00 abcd	1.68 ab	2.00	63.95 bc	100.00 a
Az. 5 + G.m. 10	11.73 abc	4.00 abcd	1.80 abcd	0.31 abcd	14.33 abcd	1.67 abcd	1.93 ab	2.00	119.80 abc	100.00 a
Az. 10 + G.m. 10	9.33 c	2.00 d	1.64 abcd	0.23 abcd	12.00 abcde	2.00 abcd	1.75 ab	2.00	76.21 bc	100.00 a
Az. 20 + G.m. 10	14.10 abc	3.90 abcd	2.18 abc	0.58 ab	16.33 a	3.00 abc	2.00 a	2.00	181.90 abc	100.00 a
Az. 5 + G.m. 20	13.27 abc	4.00 abcd	1.91 abcd	0.38 abcd	14.67 abcd	2.00 abcd	1.64 ab	2.00	205.20 abc	100.00 a
Az. 10 + G.m. 20	13.07 abc	3.57 bcd	1.91 abcd	0.37 abcd	14.67 abcd	2.00 abcd	1.68 ab	2.00	168.50 abc	100.00 a
Az. 20 + G.m. 20	11.00 bc	3.00 bcd	1.43 bcd	0.14 cd	12.00 abcde	1.67 abcd	1.34 ab	2.00	70.01 bc	100.00 a
Az. 5 + G.f. 5	11.70 abc	2.97 bcd	1.82 abcd	0.41 abcd	12.33 abcde	3.00 abc	1.43 ab	2.00	133.40 abc	100.00 a
Az. 10 + G.f. 5	18.30 a	6.47 abc	2.32 a	0.52 abcd	15.00 abc	3.00 abc	2.00 a	2.00	287.50 a	100.00 a
Az. 20 + G.f. 5	17.77 ab	7.77 a	2.28 ab	0.63 a	15.33 ab	2.33 abcd	2.00 a	2.00	211.60 abc	100.00 a
Az. 5 + G.f. 10	11.43 bc	3.00 bcd	1.81 abcd	0.32 abcd	14.00 abcde	2.00 abcd	1.99 a	2.00	106.90 abc	100.00 a
Az. 10 + G.f. 10	11.80 abc	2.57 bcd	1.67 abcd	0.25 abcd	13.00 abcde	2.33 abcd	1.83 ab	2.00	246.60 ab	100.00 a
Az. 20 + G.f. 10	9.47 c	2.23 cd	1.64 abcd	0.13 cd	11.33 abcde	1.33 abcd	1.75 ab	2.00	78.29 bc	100.00 a
Az. 5 + G.f. 20	9.47 c	1.10 d	1.59 abcd	0.23 abcd	11.00 abcde	3.00 abc	1.54 ab	2.00	143.70 abc	66.67 b
Az. 10 + G.f. 20	13.17 abc	4.00 abcd	2.23 abc	0.53abc	14.33abcd	3.67 a	2.00 a	2.00	223.30 abc	100.00 a
Az. 20 + G.f. 20	10.23 c	1.90 d	1.44 bcd	0.27abcd	11.67abcde	1.33 abcd	2.00 a	2.00	54.21 bc	100.00 a
SSP10 alone	14.50 abc	6.53 ab	2.08 abcd	0.52abcd	14.00abcde	3.33 ab	1.68 ab	2.00	123.90 abc	100.00 a
Control	12.30 abc	2.63 bcd	1.83 abcd	0.32abcd	12.33abcde	2.33 abcd	1.12 b	2.00	169.60 abc	100.00 a
Mean values	11.41	3.03	1.76	0.30	12.40	2.03	1.66	2.00	115.19	97.70
CD. ($p < 0.05$)	5.63	3.51	0.69	0.35	5.03	1.96	0.69	NS	166.20	24.78

Numbers followed by the same letter do not differ significantly at 5% level.
G.m.= Glomus mosseae, G.f.= Glomus fasciculatum, Az.= Azospirillum.

G.m. (10 g) + Az. (20 g) + SSP (10 g), which was significantly higher than G.f. (5 g) + Az. (20 g) + SSP (10 g). These two treatments were significantly superior to all other treatments including the control. The maximum increment at this stage was observed in the treatment G.f. (20 g) + Az. (10 g) + SSP (10 g).

The greatest number of new flushes/year was recorded in treatment plants inoculated with G.f. (10 g) + SSP (10 g), G.m. (10 g) + Az. (20 g) + SSP (10 g), G.f. (5 g) + Az. (10 g) + SSP (10 g), G.f. (5 g) + Az. (20 g) + SSP (10 g), G.f. (20 g) + Az. (10 g) + SSP (10 g) and G.f. (20 g) + Az. (20 g) + SSP (10 g) which produced the same number of flushes, followed by G.f. (10 g) + Az. (5 g) + SSP (10 g) that were statistically at par with each other. All the above treatments were significantly superior to the other treatments. There were no variations observed among the treatments with regard to the number of leaves/flush and all the treatments gave an average of two leaves. The maximum leaf area was recorded in the plants inoculated with G.f. (5 g) + Az. (10 g) + SSP (10 g), followed by G.f. (10 g) + Az. (10 g) + SSP (10 g). These two treatments were at par with each other and were relatively superior to all other treatments, including the control.

A 100% survival rate was observed in all treatments except G.f. (20 g) + Az. (5 g) + SSP (10 g), which had only a 66.67% survival rate.

Biomass and root characters

All mean data related to the characters of shoots and roots of one-year-old mangosteen seedlings are presented in Table 2. The maximum fresh weight of shoot was recorded

Table 2 - Morphological characters of twelve-month stage mangosteen (*Garcinia mangostana* L.) seedlings inoculated with arbuscular mycorrhizal fungi (AMF) and *Azospirillum*

Treatments (g)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)	Root to shoot dry weight ratio	Root length (cm)	Primary roots (No)	Secondary roots (No)	Tertiary roots (No)	Total roots (No)
G.m. 5	6.00 ij	1.00 lmn	3.13 kl	0.65 ij	0.21 b	10.2 k	12.0 kl	16.0 g	9.0 no	37.0 l
G.m. 10	3.05 lmn	0.89 mno	0.97 r	0.35 mno	0.36 b	23.2 efgh	21.0 fghijk	36.0 d	15.0 jklm	72.0 ghij
G.m. 20	4.79 jkl	1.08 lm	0.22 t	0.51 jklm	2.62 a	22.8 efgh	31.0 abcde	43.0 cd	19.0 ijk	93.0 efgh
G.f. 5	3.09 lmn	0.70 op	1.20 q	0.31 nop	0.26 b	20.7 hi	14.0 jkl	31.0 def	12.0 lmno	57.0 ijkl
G.f. 10	10.54 f	2.05 g	4.74 h	0.86 g	0.18 b	23.2 efgh	22.0 efghij	38.0 cd	10.0 mno	70.0 ghijk
G.f. 20	7.54 hi	1.56 i	3.27 k	0.68 hi	0.21 b	24.6 cdefgh	26.0 defghi	39.0 cd	26.0 gh	91.0 efgh
Az. 5	1.53 n	0.42 qr	0.67 s	0.21 opq	0.30 b	17.6 ij	19.0 hijkl	28.0 defg	11.0 lmno	58.0 ijkl
Az. 10	3.94 klm	1.06 lm	1.81 op	0.50 jklm	0.28 b	21.2 ghi	20.0 ghijk	32.0 def	15.0 jklm	67.0 hijkl
Az. 20	3.36 lm	0.58 pq	1.19 q	0.17 pq	0.14 b	20.3 hi	15.0 jkl	19.0 efg	8.0o	42.0 jkl
Az. 5 + G.m. 5	5.57 jk	1.13 kl	1.94 o	0.38 lmn	0.20 b	18.3 ij	20.0 ghijk	32.0 def	14.0 klmn	66.0 hijkl
Az. 10 + G.m. 5	8.32 gh	1.83 h	3.77 j	0.90 g	0.24 b	21.5 ghi	23.0 defghij	38.0 cd	24.0 hi	85.0 fghi
Az. 20 + G.m. 5	6.42 ij	1.37 ij	2.55 m	0.55 ijk	0.22 b	14.6 j	19.0 hijkl	52.0 bc	30.0 g	101.0 defg
Az. 5 + G.m. 10	11.04 ef	2.51 f	4.79 h	1.10 f	0.23 b	20.3 hi	29.0 bcdefg	86.0 a	46.0 de	161.0 b
Az. 10 + G.m. 10	5.56 jk	1.49 ij	2.21 n	0.57 ijk	0.26 b	26.8 bcde	36.0abc	94.0 a	58.0 ab	188.0 a
Az. 20 + G.m. 10	12.52 de	2.40 f	5.90 f	1.16 f	0.20 b	27.4 bcd	30.0bcdef	59.0 b	38.0 f	127.0 cd
Az. 5 + G.m. 20	2.49 mn	0.26 r	0.92 r	0.11 q	0.11 b	16.2 j	10.0 l	18.0 fg	12.0 lmno	40.0 kl
Az. 10 + G.m. 20	4.08 klm	0.83 no	1.68 p	0.36 lmno	0.21 b	21.7 fghi	22.0 efghij	33.0 def	14.0 klmn	69.0 hijk
Az. 20 + G.m. 20	8.47 gh	1.95 gh	3.69 j	0.81 gh	0.22 b	28.4 bc	16.0 jkl	44.0 cd	16.0 jkl	76.0 ghi
Az. 5 + G.f. 5	7.72 hi	1.30 jk	3.00 l	0.52 ijkl	0.17 b	22.5 fgh	20.0ghijk	34.0 de	20.0 ij	74.0 ghi
Az. 10 + G.f. 5	19.72 b	6.31 a	10.55 b	3.03 a	0.29 b	36.4 a	40.0 a	58.0 b	60.0 a	158.0 b
Az. 20 + G.f. 5	18.60 b	3.32 de	6.20 e	1.42 e	0.23 b	23.2 efgh	18.0 ijkl	28.0 defg	30.0 g	76.0 ghi
Az. 5 + G.f. 10	11.36 ef	2.49 f	5.39 g	1.36 e	0.25 b	25.8 cdef	28.0 cdefgh	40.0 cd	47.0 de	115.0 cde
Az. 10 + G.f. 10	19.65 b	2.49 f	8.76 c	2.15 c	0.25 b	18.2 ij	15.0 jkl	33.0 def	42.0ef	90.0 efgh
Az. 20 + G.f. 10	9.80 fg	3.97 c	4.49 i	1.82 d	0.41 b	23.2 efgh	16.0 jkl	30.0 defg	18.0 jk	64.0 hijkl
Az. 5 + G.f. 20	16.95 c	4.01 c	6.92 d	1.67 d	0.24 b	26.8 bcde	32.0 abcd	43.0 cd	50.0 cd	125.0 cd
Az. 10 + G.f. 20	25.53 a	3.39 d	11.09 a	2.56 b	0.23 b	30.2 b	30.0 bcdef	42.0 cd	53.0 bc	125.0 cd
Az. 20 + G.f. 20	13.91 d	5.23 b	5.43 g	1.35 e	0.25 b	23.8 defgh	29.0 bcdefg	38.0 cd	43.0 ef	110.0 def
SSP10 alone	16.27 c	3.15 e	6.89 d	1.79 d	0.26 b	30.4 b	38.0 ab	44.0cd	58.0 ab	140.0 bc
Control	10.54 f	3.91 c	3.65 j	0.45 klmn	0.12 b	25.4 cdefg	28.0 cdefgh	32.0 def	18.0 jk	78.0 ghi
Mean values	9.60	2.16	4.04	0.98	0.31	22.93	23.41	40.0	28.138	91.552
C.D. ($p< 0.05$)	1.63	0.19	0.19	0.15	0.33	3.60	8.17	13.08	4.903	26.15

Numbers followed by the same letter do not differ significantly at 5% level.
G.m.= *Glomus mosseae,* G.f.= *Glomus fasciculatum,* Az= *Azospirillum.*

in the plants inoculated with G.f. (20 g) + Az. (10 g) + SSP (10 g), which was significantly superior to all other treatments. This was followed by G.f. (5 g) + Az. (10 g) + SSP (10 g), G.f. (10 g) + Az. (10 g) + SSP (10 g) and G.f. (5 g) + Az. (20 g) + SSP (10 g). These three treatments were at par with each other and significantly superior to all the other treatments including the control. The highest fresh weight of the root was recorded in the treatment G.f. (5 g) + Az. (10 g) + SSP (10 g) followed by G.f. (20 g) + Az. (20 g) + SSP (10 g). The differences between the treatment means were significant and both these treatments were also significantly superior to all other treatments including the control.

Maximum dry weight of shoot and root were recorded in plants inoculated with G.f. (20 g) + Az. (10 g) + SSP (10 g) and G.f. (5 g) + Az. (10 g) + SSP (10 g), followed respectively by G.f. (5 g) + Az. (10 g) + SSP (10 g) and G.f. (20 g) + Az. (10 g) + SSP (10 g). In both cases (i.e. dry weight of shoot and root), the means of the two treatments which gave maximum dry weight not only significantly differed between them but was also superior to all other treatments. A critical analysis revealed that only the relative positions of the best and second-best treatments in the case of root and shoot dry weight inter changed.

Maximum dry weight ratio of roots and shoots were recorded in plants inoculated with G.m. (20 g) + SSP (10 g), which was significantly superior to all other treatments, which were at par with each other including the control.

The maximum root length was observed in plants inoculated with G.f. (5 g) + Az. (10g) + SSP (10 g), which was significantly superior to all other treatments. This was followed by plants treated with SSP (10 g) alone and G.f. (20 g) + Az. (10 g) + SSP (10 g), which were statistically at par. These treatments produced better root length, giving significantly superior results compared to all other remaining treatments including the control.

Maximum and significantly higher primary root count were recorded in the plants inoculated with G.f. (5 g) + Az. (20 g) + SSP (10 g) followed by SSP (10 g) alone. The greatest number of secondary roots was observed in the plants inoculated with G.m. (10 g) + Az. (10 g) + SSP (10 g), which was at par with G.m. (10 g) + Az. (5 g) + SSP (10 g), the next best treatment and significantly superior to all other treatments. This was followed by G.f. (5 g) + Az. (10 g) + SSP (10 g) and G.m. (5 g) + Az. (20 g) + SSP (10 g). The means of the latter two treatments were at par with each other. The highest tertiary root count was observed in the treatment G.f. (5 g) + Az. (10 g) + SSP (10 g), which was statistically at par with the treatment combination G.m. (10 g) + Az. (10 g) + SSP (10 g) and SSP (10 g) alone. The latter two treatments were at par with G.f. (20 g) + Az. (10 g) + SSP (10 g). The above treatments were significantly higher than all other treatments including control.

The total number of roots was greatest in plants inoculated with G.m. (10 g) + Az. (10 g) + SSP (10 g) , which was superior to all other treatments. This was followed by G.m. (10 g) + Az. (5 g) + SSP (10 g), which was at par with G.f. (5 g) + Az. (10 g) + SSP (10 g) and SSP (10 g) alone. The means of these treatments were significantly superior to the means of all other treatments including the control.

Biochemical characters of seedling leaves

For the purpose of analyzing the biochemical characters, the treatments were categorized as those showing the best growth, intermediary growth and the least growth (Fig. 1). Typical treatments for each group were selected and are presented in Table 3: best growth, G.f. (5 g) + Az. (10 g) +

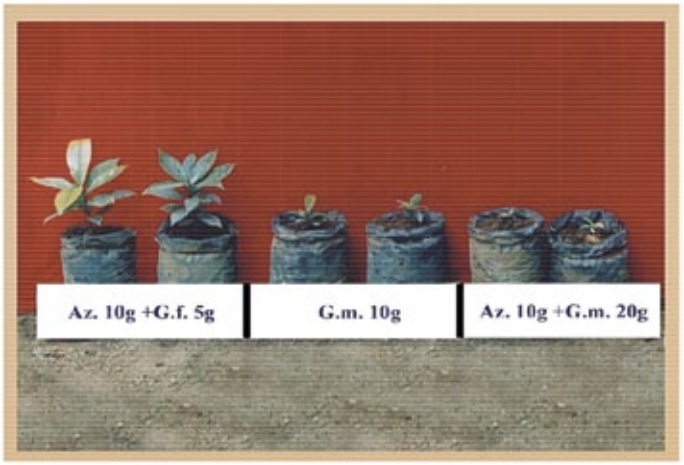

Fig. 1 - Seedling of treatments, showing the best, the intermediate and the least growth response to arbuscular mycorrhizal fungi (AM fungi) and *azospirillum* in Mangosteen (*Garcinia mangostana* L.).

Table 3 - Biochemical characters of the leaf samples in one-year-old mangosteen (*Garcinia mangostana* L.) seedlings treated with arbuscular mycorrhizal fungi and *Azospirillum*

AMF and *Azospirillum* inoculated plants	N (%)	P (%)	K (%)	Crude protein (%)	Na (%)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Chlorophyll a/b ratio	Total chlorophyll (mg/g)	Total phenols (mg/g)	Total carbohydrates (mg/g)	Abscisic acid (mg/g)
Best (Az. 10 + G.f. 5)	2.35	0.19 a	0.34	14.67 a	0.34	0.95 a	0.42 a	2.30	1.37 a	0.64	7.85 a	0.21 a
Intermediate (G.m. 10 g)	2.08	0.05 b	0.30	12.99 b	0.34	0.66 b	0.29 a	2.38	0.95 ab	0.46	6.59 ab	0.16 ab
Least (Az. 10 + G.m. 20)	1.79	0.04 b	0.28	11.22 c	0.34	0.17 c	0.11 b	1.88	0.27 b	0.47	4.85 b	0.08 b
Control	1.94	0.04 b	0.29	12.10 bc	0.34	0.41 c	0.20 ab	2.05	0.61 ab	0.46	5.72 ab	0.14 ab
Mean values	2.04	0.08	0.30	12.75	0.34	0.55	0.25	2.15	0.80	0.51	6.25	0.15
C.D. ($p<0.05$)	NS	0.09	NS	1.46	NS	0.25	0.17	NS	0.80	NS	2.13	0.096

Numbers followed by the same letter do not differ significantly at 5%.
G.m.= *Glomus mosseae,* G.f.= *Glomus fasciculatum,* Az= *Azospirillum.*

SSP (10 g); intermediate growth, G.m. (10 g) + SSP (10 g); least growth, G.m. (20 g) + Az. (10 g) + SSP (10 g).

The nitrogen content was highest in the maximum growth treatment G.f. (5 g) + Az. (10 g) + SSP (10 g). A distinct trend was noticed, with maximum values found in treatments with the best growth, the intermediary values being recorded in treatments showing intermediate growth, and the lowest values in treatments showing the least growth. However, there were no significant differences observed between the treatment means.

In the case of phosphorus, a pattern nearly similar to that of nitrogen was observed. However, the mean content in the treatment, which showed the best growth, was significantly superior to other treatments including the control.

The potassium content also revealed a similar trend: the treatment with maximum growth also had the highest potassium content. However there were no significant differences observed between the various treatment means including the control. The control treatment gave the values between intermediate growth and least value treatments.

The highest crude protein content was also observed in the treatment which showed best growth. It was significantly superior to other treatments and also to the control. The treatments which showed intermediate growth also showed higher values for this parameter and they were significantly higher than the treatment with least growth and control plants.

Treatments showing the best, intermediate and least growth, as well as the control, all showed the same level of sodium content.

Maximum chlorophyll a and b and total chlorophyll content were observed in the treatment that recorded the maximum growth. In the case of chlorophyll a, this was significantly higher than the means observed in plants of other categories and the control. The content of the plants with the least growth was at par with control. In the case of chlorophyll b content , the trend observed was similar to that of growth. The treatment with maximum growth had the highest chlorophyll b content, which was on par with the treatment showing intermediate growth. These two levels were significantly superior to the level observed in the plants with the least growth, but it was statistically at par with the control.

Contrary to what is commonly believed, the total phenol content was highest in the treatments with maximum growth (Fig. 2). However, there were no significant differences observed between treatment means and the control.

The greatest amount of total carbohydrate was found in the treatment that had maximum growth. This was at par with the treatment means that showed intermediate growth and also with the control.

The content of abscisic acid, which normally goes hand in hand with growth inhibition, was also contrary to normal lines of thought. The treatments which revealed more growth also had the highest abscisic acid content. The means of the group with the highest abscisic acid content were also at par with those of the treatment with intermediate growth and the control, but the treatment means of the first group was significantly superior to the category which showed the least growth (Fig. 1, 2, and 3).

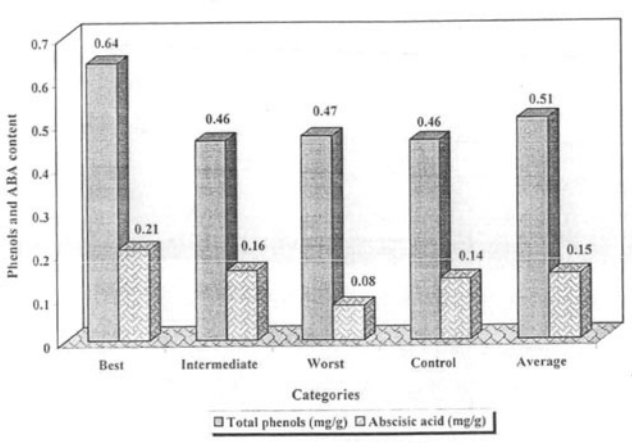

Fig. 2 - Total phenols and abscisic acid content of the AM fungi and Azospirillum treated plants showing best, intermediate and least growth in mangosteen (Garcinia mangostana L.).

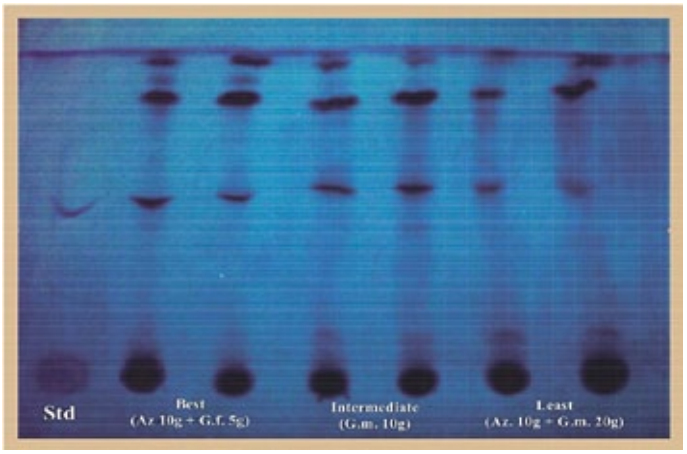

Fig. 3 - Banding pattern observed in TLC used to quantify abscisic acid content in various treatments using arbuscular mycorrhizal fungi (AM fungi) and *azospirillum* in Mangosteen (*Garcinia mangostana* L.)

Microbial population and percent root infection

The mean data related to microbial population and the percent root infection by AM fungi are presented in Table 4.

Total spore population of arbuscular mycorrhizal fungi (AMF)

The highest number of spores was recorded in the rhizosphere of plants inoculated with G.f. (20 g) + SSP (10 g), followed by G.f. (20 g) + Az. (20 g) + SSP (10 g). These two treatments were on par with all other treatments except the control, proving the superiority of the treatment in colonization.

Percent root infection

Root infection, which gave a clear picture of colonization, was also studied. The highest percentage of root infection was recorded in plants inoculated with G.f. (5 g) + Az. (10 g) + SSP (10 g) and G.f. (20 g) + Az. (10 g) + SSP (10 g), with both registering the highest values of mean infection percentage. These treatments were at par with all other treatments except G.m. (5 g) + SSP (10 g),

Table 4 - Microbial population in seedlings rhizosphere and infectivity in roots after one year of inoculation in mangosteen (*Garcinia mangostana* L.)

Treatments	Total AMF Spores (No./100 g of soil)	Root Infection by AMF (%)	*Azospirillum* population (x 106 cfu/g of soil)
G.m. 5	198.00 ab	40.00 cdef	0.22 bc
G.m. 10	214.00 ab	55.00 abcdef	0.24 bc
G.m. 20	219.00 ab	65.00 abcde	0.26 abc
G.f. 5	206.00 ab	40.00 cdef	0.27 ab
G.f. 10	222.00 ab	60.00 abcdef	0.28 ab
G.f. 20	228.00 a	70.00 abcd	0.30 ab
Az. 5	136.00 bc	35.00 def	0.34 ab
Az. 10	142.00 abc	40.00 cdef	0.35 ab
Az. 20	148.00 abc	30.00 ef	0.37 ab
Az. 5 + G.m. 5	200.00 ab	45.00 bcdef	0.31 ab
Az. 10 + G.m. 5	213.00 ab	55.00 abcdef	0.36 ab
Az. 20 + G.m. 5	215.00 ab	50.00 abcdef	0.37 ab
Az. 5 + G.m. 10	204.00 ab	50.00 abcdef	0.33 ab
Az. 10 + G.m. 10	218.00 ab	65.00 abcde	0.37 ab
Az. 20 + G.m. 10	222.00 ab	70.00 abcd	0.34 ab
Az. 5 + G.m. 20	206.00 ab	60.00 abcdef	0.32 ab
Az. 10 + G.m. 20	220.00 ab	75.00 abc	0.38 ab
Az. 20 + G.m. 20	224.00 ab	55.00 abcdef	0.39 ab
Az. 5 + G.f. 5	204.00 ab	80.00 ab	0.32 ab
Az. 10 + G.f. 5	216.00 ab	90.00 a	0.36 ab
Az. 20 + G.f. 5	219.00 ab	85.00 ab	0.40 a
Az. 5 + G.f. 10	210.00 ab	80.00 ab	0.33 ab
Az. 10 + G.f. 10	220.00 ab	85.00 ab	0.37 ab
Az. 20 + G.f. 10	224.00 ab	85.00 ab	0.38 ab
Az. 5 + G.f. 20	213.00 ab	85.00 ab	0.37 ab
Az. 10 + G.f. 20	224.00 ab	90.00 a	0.35 ab
Az. 20 + G.f. 20	226.00 ab	80.00 ab	0.39 ab
SSP10 alone	150.00 abc	30.00 ef	0.23 bc
Control	90.00 c	25.00 f	0.12 c
Mean values	201.10	61.21	0.32
C.D. ($p< 0.05$)	73.55	32.69	0.14

Numbers followed by the same letter not differ significantly at 5% level.
G.m= *Glomus mosseae,* G.f= *Glomus fasciculatum*.

G.f. (5 g) + SSP (10 g), all the *Azospirillum* + SSP alone treated plants, G.m. (5 g) + Az. (5 g) + SSP (10 g), SSP (10 g) and also the control.

Azospirillum population in soil

The maximum *Azospirillum* population was recorded in the rhizosphere of plants inoculated with G.f. (10 g) + Az. (20 g) + SSP (10 g), which was on par with all other treatments except the combination of G.m. (5 g) and (10 g) alone with SSP (10 g) absolute control of SSP (10g) alone and the control plants.

4. Discussion and Conclusions

The best treatments in case of microbial inoculation was a combination of G.f. (5 g) + Az. (10 g) + SSP (10 g) followed by G.f. (5 g) + Az. (20 g) + SSP (10 g).

Critical analysis of the data revealed that the percentage of AM fungi infection was the highest in treatments with maximum growth. The next best in order of growth also showed a high infection percentage. Though the total spore count was not the highest the treatments were on par with the treatments that yielded the highest spore count namely G.f. (20 g) treated plants. These two-treatment combinations also showed a high *Azospirillum* population. These three characters proved, beyond a doubt, that the combination was best among the treatments for maximum growth. This is a reflection of mycelial mat formation on absorbing roots, which in turn leads to higher P, N and K uptake as observed in the study. The effects were most pronounced with regard to P as the content registered a three-fold increase. This should have been due to two factors, namely the external apply of SSP (10 g) and a proven concept that mycorrhiza gives out an organic acid secretion which is capable of solubilising as well as mobilizing the acid soluble phos-

phate (i.e. semi-soluble phosphate) into soluble form. Some of the previous reports substantiate these findings (Bartlett and Lewis, 1973). Increases in selective uptake of major nutrients have also been reported by several workers (Hatch, 1937; Umesh *et al.,* 1988; Rizzardi, 1990). The capability of transferring it into the soluble form has also been reported (Bolan *et al*., 1984). Another possible reason is that they can enhance the storage capacity, and the continuous disintegration of arbuscules leads to the availability of more mineral nutrients to the host (Gerdemann, 1968).

The higher levels of P, almost three times, should have been the reason for quantitative improvement of the root characters. This can be observed in the best treatment which induced the highest fresh and dry weight of roots, as well as characters such as length of longest root and improved characters of number of roots. In mangosteen, Masri and Azizah (1998) reported alternation of root characters such as root density and branching density, which led to 67-88% higher uptake of P. Studies on alteration of rooting density are of paramount importance in mangosteen owing to the fact that the crop produces only magnolioid roots, an unique feature of mangosteen among other fruit crops. Due to lack of production of root hairs, absorption of nutrients and water is less and even survival at nature's mercy or benevolence. As such any treatment, which improves the qualitative aspects of rooting such as root length, root branching and root density will certainly influence all aspects of growth and productivity and hence should be the prime consideration in crop management.

The production of growth promoting substances by *Glomus mosseae* and *Glomus fasciculatum* is well documented (Miller, 1971; Crafts and Miller, 1974; Slankis, 1975). Greater nutrient uptake and growth promoting substances should have been the reason for the increased chlorophyll content and higher leaf area, which together accounted for higher carbon assimilation (Estrada-Luna *et al*., 2000) leading to higher carbohydrate content as observed in the study. Higher crude protein content also points to the level of protein synthesis due to AM fungi inoculation, which is a new area worth to be probed further.

The greater nutrient uptake and carbohydrate accumulation resulted in greater shoot and root biomass and dry weight content. Differences observed in growth increment during intervals of observation are basically due to infectivity and colonization of AM fungi on feeder roots. This is partly an efficiency factor of the fungi and secondly is influenced by soil ecology factors as well as the host. In mangosteen, Masri *et al.* (1998) observed increased growth due to AM fungi inoculation and thereby reduced nursery period for mangosteen.

As in the case of activation of growth using bioregulators, here also the content of inhibitors - namely phenols and ABA - were higher in the best treatment and a general decrease was observed in the treatment showing the least growth. This can also be argued only on the lines of correlative inhibition. The higher content did not inhibit the growth, as the balance of various growth regulators should have been more towards the plant growth promoters than growth inhibitors. More detailed investigation encompassing the whole endogenous levels of plant hormones at the critical stages of bud dormancy, bud activation, flushing and post flushing can only answer this vital question.

The present study convincingly proves the efficacy of the two treatments in activating growth of mangosteen. The mean values of growth characters obtained in the best treatments are far superior to the selection indices standardized for seedling growth (Yusuf and Kurien, 2012). This investigation is of great practical application and gains more importance as mangosteen is a crop which lacks fine root hairs (Richards, 1990) while the study revealed that colonization of AM fungi and *Azospirillum* takes place on the network of fine ramified roots enabling better uptake of nutrients and thereby growth.

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FERGUSON A.R., BOLLARD E.G., 1990 - *Domestication of the kiwifruit*, pp. 165-246. - In: WARRINGTON I.J., and G.C. WESTON (eds.) *Kiwifruit: Science and Management*. Ray Richards Publisher in assoc. with the N.Z. Soc. Hort. Sci., Auckland, pp. 576.

<u>*Book*</u>

NICKELL L.G., 1983 - *Plant growth regulating chemicals*. - Vol. I. CRC Press, Boca Raton, Florida, USA, pp. 280.

<u>*Thesis and Dissertations*</u>

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<u>*Bulletin or Report*</u>

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