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Identification of endophytic bacteria in chickpea (*Cicer arietinum* L.) and their effect on plant growth*

R. RANGESHWARAN[1], J. RAJ[2] and P. SREERAMA KUMAR[1]

[1]Project Directorate of Biological Control, Post Bag No. 2491, H. A. Farm Post, Bellary Road, Bangalore – 560024, Karnataka, India.

[2]Department of Agricultural Microbiology, University of Agricultural Sciences, Bangalore – 560065, Karnataka, India.

E-mail: rangeshw@rediffmail.com

ABSTRACT: **Five endophytic bacteria were isolated from healthy chickpea (*Cicer arietinum L.*) plants by surface-disinfestation method and identified based on morphological, physiological and biochemical tests. *Erwinia herbicola* and *Enterobacter agglomerans*, which had nitrate-solubilizing ability, were isolated from the root endosphere and *Bacillus megaterium*, *B. circulans* and one unidentified species of *Bacillus* were isolated from the leaf and stem tissues. None of the isolates had phosphate-solubilizing ability. All the isolates tolerated a wide range of pH and exhibited growth from pH 5 to 9. *B. circulans* and *E. agglomerans* tolerated an alkaline pH of 11, which is unusual. Higher growth promotion was noticed in chickpea plants treated with *B. megaterium*, *E. agglomerans* and *Bacillus* sp. and the plants treated with endophytes survived well in the presence of the wilt pathogen, *Rhizoctonia solani*. Seedlings from seeds treated with bacteria showed an increase in phenol content up to sixth day after inoculation. The maximum phenol content (483.33μg g^{-1}) was noticed in *B. megaterium* treated and the lowest (246.67μg g^{-1}) was in control. Endophytic bacteria from healthy plant tissue could play a useful role in plant protection.**

KEY WORDS: Chickpea, endophytic bacteria, growth promotion, identification

INTRODUCTION

Endophytic bacteria colonize plant tissues internally and are involved in improving plant health. They are ubiquitous and occur in a broad spectrum of plant species (Hallmann, 2001). These bacteria can move systemically throughout the plant and the association can either be neutral to the plant or positive when plant growth and / or health are stimulated. Most endophytic bacteria are probably found in the intercellular spaces of the root cortex or stem where they can occur in high densities (Chen *et al.*, 1995; Sturz and Matheson, 1996). Recently, interest in endophytic bacteria has increased especially for those bacteria having commercial applications such as plant growth promotion and stimulation of plant defence mechanisms. The best characterized plant endophytic interactions are those of the nitrogen-fixing bacteria, like the rhizobium-legume symbiosis

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or the symbiosis between free-living N-fixing bacteria. These endophytic bacteria improve the plant's nitrogen status, but do not directly affect plant health, except that a stronger plant can better resist attack by plant pathogens. Endophytic bacteria are commonly isolated from internal plant tissue either directly by centrifugation or pressure bomb extraction as well as directly following disinfestation of the plant surface (Hallmann *et al.*, 1997; McInroy and Kloepper, 1995a and 1995b).

The genera of endophytic bacteria that have been isolated from various crops include *Bacillus, Pseudomonas, Burkholderia, Erwinia, Clavibacter, Xanthomonas, Phyllobacterium, Enterobacter, Flavobacterium, Agrobacterium, Corynebacterium, Curtobacter, Pantoea, Micrococcus* and *Lactobacillus* (Hallmann, 2001).

In India, no reports are available on the identification of endophytic bacteria from chickpea. Sharma *et al.* (2005) reported bacteria belonging to the *Rhizobium* and *Agrobacterium* group as wheat root endophytes. Ramteke *et al.* (2004) reported the phosphate solubilizing ability of endophytic bacteria in sugarcane and importance of endophytic bacteria in control of wilt pathogens was indicated (Rangeshwaran *et al*, 2002). In the present study, an attempt was made to isolate and identify endophytic bacteria from healthy chickpea tissue and also to see their effect on chickpea growth.

MATERIALS AND METHODS

Isolation of endophytic bacteria

Endophytic bacteria from healthy chickpea plants were isolated by the procedure suggested by McInroy and Kloepper (1995a). Healthy chickpea of different varieties were uprooted and packed into new polythene covers. The plant samples were transported immediately to the laboratory. Root and stem samples (2-3cm long) were drawn using a sterile scalpel. For younger plants (14 days), root samples were taken just below the soil line and 5-10cm below the soil line were taken for older plants (21days). Stem samples were taken 1-2 cm above the soil line in younger plants and 10cm above the soil line in older plants. Stem samples were weighed and surface sterilized with 20% hydrogen peroxide for 10 minutes and rinsed four times with 0.02 M potassium phosphate buffer (pH7.0). Root samples were surface disinfected with 1.05% sodium hypochlorite and washed in four changes of 0.02M phosphate buffer solution. Measured quantity of 0.1ml aliquot from the final buffer wash was removed and transferred in 9.9ml Tryptic-Soya broth to serve as sterility check. Samples were discarded if growth was detected in the sterility check within 48h. Selected samples were triturated in 9.9ml of buffer in a sterile pestle and mortar. The triturate was serially diluted in potassium phosphate buffer solution. The dilutions were poured on plates containing sterilized Tryptic Soya Agar (TSA). Representative colonies (based on colony morphology) were transferred to fresh TSA plates to establish pure cultures. Shake cultures of purified strains were prepared at room temperature for 18-24h in Tryptic Soya Broth (TSB) and centrifuged at 5000rpm for 7 minutes. Pellets were suspended in 2.0ml TSB and resuspended in (1: 1 ratio) 20% glycerol and maintained at –80°C in cryovials for later identification.

Identification of selected bacteria

Five selected endophytic bacteria were identified. The isolates were subjected to various tests like Gram's reaction, morphological tests, physiological and biochemical tests, as outlined below. The identification of the isolates was accorded the relevant MTCC numbers by Institute of Microbial Technology (IMTECH), Chandigarh.

Phosphate solubilization in solid medium

The endophytic bacteria were tested for their ability to solubilize phosphate in Sperber's agar medium (Sperber, 1957) and Pikovskaya's agar medium (Pikovskaya's, 1948). In this experiment Sperber's medium and Pikovskaya's medium were poured onto previously sterilized Petri plates. After solidification, 5mm discs of bioagents were placed at the centre of the plates and incubated at 30°C. For comparison, standard phosphate solubilizing bacterium *Bacillus megaterium* was also inoculated at the centre of the media. Three replications were

maintained for each treatment and incubated at 27 ± 1°C. The relative growth and solubilization zones were recorded at 24-hour intervals for 5 days.

Culturing and growth promotion test

The isolated bacteria were initially screened for plant growth promoting ability in potted chickpea plants under sterile conditions. Cultures were multiplied in 100ml Tryptic Soya Broth (TSB) on a shaker at 150rpm for 48h. Cells were harvested by centrifuging at 7000rpm for 15 minutes and suspended in phosphate buffer (100ml). Seeds were first sterilized in 0.1% mercuric chloride, washed and treated by dipping in the buffer having the suspended cells. Number of viable cells per seed was determined by serially diluting on TSA plates. The treated seeds were sown in pots (washed) containing 4kg sterile field soil. Observations were recorded at 30 days. In another experiment, the treated seeds were sown in pots that were already inoculated with the wilt pathogen *Rhizoctonia solani* and observations were recorded at 14 days. Observations on per cent germination, root length and shoot length were recorded and vigour index was calculated.

Estimation of phenol content

Chickpea leaves (1g) were homogenized in 10ml of 80% methanol and agitated for 15 minutes at 70°C. One ml of the methanolic extract was added to 5ml of distilled water and 250µl of Folin-Ciocalteau reagent (1N) was added and the solution was kept at 25°C. After 3min, 1ml of saturated solution of Na_2CO_3 and 1 ml of distilled water added and the reaction mixture was incubated for 1h at 25°C. The absorption of the developed blue colour was measured using a spectrophotometer at 725nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reaction with phenol and expressed as phenol equivalents in µg g^{-1} fresh weight (Meena *et al.*, 2000).

Mass culturing of pathogen

Rhizoctonia solani was mass cultured in autoclaved wheat bran + vermiculite (1: 1) mixture containing 10% glucose. The mixture was first thoroughly mixed and moisture adjusted to 15 - 20%. The inoculum was obtained by growing the pathogens in Potato Dextrose Broth (PDB) and added to the mixture @ 5%. Autoclavable plastic bags (Himedia) were used for the process. The mixture was used @ 5g kg^{-1} of soil. The endophytes were compared with two proven plant growth-promoting rhizobacteria (PGPR), *viz.*, *Pseudomonas fluorescens* (PDBCAB2) and *Bacillus subtilis* (PDBCABN22), which were obtained from the culture collection of Project Directorate of Biological Control (PDBC), Bangalore.

RESULTS AND DISCUSSION

Identification of the endophytic bacteria and strain designation

Five endophytic bacteria were isolated from healthy chickpea plants. The isolates were temporarily designated as UASB1, UASB2, UASB3, UASB4 and UASB5. The isolates UASB4 and UASB5 were isolated from the root endosphere and the others were from the leaf and stem tissue. The five endophytic bacteria were identified based on morphological, physiological and biochemical tests. Density was translucent for all and only UASB5 produced yellowish pigment. The isolates UASB1, UASB2 and UASB3 were gram-positive, whereas UASB4 and UASB5 were gram-negative and all were short to moderate-sized rods with single flagellar arrangement. Endospore was present in the Gram-positive ones; UASB1 had a central spore and UASB2 and UASB3 had sub-terminal arrangement. The sub-terminal spores were oval with sporangia bulging. All the endophytes were motile and no fluorescence in UV was detected.

The physiological tests were conducted for growth at different temperatures, pH, and NaCl concentration and for anaerobic growth (Table 1). The isolate UASB5 grew at 10°C and all isolates except UASB3 could grow at 15°C. Maximum temperature tolerated for growth was 42°C except for UASB4, which could only grow up to 37°C. All the isolates surprisingly tolerated a wide range of pH and growth was observed from pH 5.0 to 9.0. UASB3 and UASB4 tolerated an alkaline pH of 11.0, which is unusual. UASB1 and 3 tolerated 7 per cent

Table 1. Physiological tests conducted for identification of bacteria

Tests	Results				
Growth at temp	UASB1	UASB2	UASB3	UASB4	UASB5
(°C)					
4	-	-	-	-	-
10	±	±	-	-	+
15	+	+	±	+	+
25	+	+	+	+	+
30	+	+	+	+	+
37	+	+	+	+	+
42	+	+	+	-	+
55	-	-	-	-	-
65	-	-	-	-	-
Growth at pH					
5.0	+	+	+	+	+
5.7	+	+	+	+	+
6.8	+	+	+	+	+
8.0	+	+	+	+	+
9.0	+	+	+	+	+
11.0	-	±	+	+	±
Growth on NaCl (%)					
2.5	+	+	+	+	+
5.0	+	±	+	+	+
7.0	+	-	+	+	±
8.5	±	-	+	-	-
10.0	-	-	±	-	-
Growth under anaerobic conditions	±	+	-	+	+

- = Growth absent; + = Growth present; ± = Poor growth

NaCl and the rest could grow at 5 per cent NaCl in the medium. The isolates UASB2, UASB4 and UASB5 could also grow under anaerobic conditions.

Various biochemical tests were conducted for the five endophytic isolates (Table 2). The isolates UASB4 and UASB5 could grow on MacConkey agar and only UASB5 was positive for indole and Voges Proskauer test. UASB2 tested positive for methyl red. All the isolates were positive for citrate utilization, catalase activity, gelatin hydrolysis and arginine dihydrolase tests. None of them was positive for H_2S or ornithine decarboxylase and also no isolates produced gas from glucose. The Gram-positive isolates were positive for casein hydrolysis, starch hydrolysis and cytochrome oxidase. Only UASB2 could hydrolyse urea, but UASB3, UASB4 and UASB5 could reduce nitrate, whereas UASB4 could also reduce nitrite. UASB3 showed oxidative type of fermentation, whereas UASB4 and UASB5 were fermentative type. Acid production from different carbohydrate sources was tested for all the endophytic isolates (Table 3). The isolates UASB1 and UASB2 could produce acid only from dextrose, melibiose and trehalose. UASB3 did not produce acid from adonitol, dulcitol and rhamnose, whereas UASB4 could not produce acid from adonitol and dulcitol only. UASB5 was positive for all the carbohydrates tested except for adonitol. In the test for phosphate solubilizing ability, none of the endophytes was positive which was unusual.

Based on the above tests, the endophytic bacteria were identified as *Bacillus megaterium* (UASB1), *Bacillus* sp. (UASB2), *Bacillus circulans*

Table 2. Biochemical tests conducted for identification of bacteria

Test	Results				
	UASB1	UASB2	UASB3	UASB4	UASB5
Growth on MacConkey Agar	-	-	-	+	+
Indole test -	-	-	-	+	
Methyl red test	-	+	-	-	-
Voges Proskauer test	-	-	-	-	+
Citrate utilization	+	±	+	+	+
Gas production from Glucose	-	-	-		-
Casein hydrolysis	+	+	+	-	-
Starch hydrolysis	+	+	+	-	-
Urea hydrolysis	-	+	-	-	-
Nitrate reduction	-	-	+	+	+
Nitrite reduction	-	-	-	+	-
H_2S production	-	-	-	±	-
Cytochrome oxidase	+	+	+	-	-
Catalase test+	+	+	+	+	
Oxidation/Fermentation (O/F)	-	-	O	F	F
Gelatin hydrolysis	+	+	+	+	+
Arginine dihydrolase	+	±	+	+	+
Lysine decarboxylase	-	-	-	-	+
Ornithine decarboxylase	-	-	-	-	-

- = Growth absent; + = Growth present; ± = Poor growth; O = Oxidative ; F = Fermentative

Table 3. Acid production from carbohydrate tests conducted for identification of bacteria

Tests	Results				
	UASB1	UASB2	UASB3	UASB4	UASB5
Adonitol	-	-	-	-	-
Arabinose	-	-	+	+	+
Cellobiose	-	-	+	+	+
Dextrose	+	+	+	+	+
Dulcitol	-	-	-	-	+
Fructose	-	+	+	+	+
Galactose	-	-	+	+	+
Inositol	-	-	+	+	+
Lactose	-	-	+	+	+
Maltose	-	-	+	+	+
Mannitol	-	-	+	+	+
Melibiose	+	+	+	+	+
Raffinose	-	-	+	+	+
Rhamnose	-	-	-	+	+
Salicin	-	-	+	+	+
Sorbitol	-	-	+	+	+
Sucrose	-	-	+	+	+
Trehalose	+	+	+	+	+
Xylose	-	-	+	+	+

- = Positive; + = Negative

Table 4. Bacteria identified on the basis of the identification tests conducted and their designated MTCC number (IMTECH, Chandigarh)

Strain designation before identification	Identity	Modified strain designation	MTCC Number
UASB1	*Bacillus megaterium*	UASEBCH1	6533
UASB2	*Bacillus* sp.	UASEBCH2	6534
UASB3	*Bacillus circulans*	UASEBCH3	6535
UASB4	*Erwinia herbicola*	UASEBCH4	6720
UASB5	*Enterobacter agglomerans*	UASEBCH5	6536

(UASB3), *Erwinia herbicola* (UASB4) and *Enterobacter agglomerans* (UASB5) (Table 4). The bacteria were given modified strain numbers because of their characteristics and source of isolation were also given the required MTCC numbers by Institute of Microbial Technology (IMTECH), Chandigarh.

This is the first report on identification of endophytic bacteria in healthy chickpea (*Cicer arietinum* L.) from India. The nitrate solubilizing ability of some of the strains could be helpful in plant nutrient uptake. The phosphate solubilizing inability of one of the endophytes identified as *B. megaterium* was surprising. Endophytes could be behaving differently when inside the plant tissue. The endophyte *E. herbicola* is reported to play a role in nodulation by *Rhizobium* (Handelsman and Brill, 1985). Several reports are available on the identification of endophytes in plant tissue. In the present study, the standard surface disinfestation method was used for isolation. Pleban *et al.* (1995) reported that isolates of different endophytic bacteria were recovered from surface-disinfected seeds obtained from commercial companies, plants in the field and tissue culture. The bacteria were isolated from seeds after stringent surface-disinfection. The work on isolation and identification of endophytic bacteria was recently started and many publications reported only the isolation and identification of endophytic bacteria (McInroy and Kloepper, 1991; Fisher *et al.*, 1992).

Assay for growth promoting ability

The selected endophytic and rhizospheric bacteria were tested for their important character of growth promoting ability of seed treated chickpea plants in unsterile soil. All the tested bacteria showed positive effect on plant growth (Table 5). Higher growth promotion was noticed in *B. megaterium, E. agglomerans,* and *Bacillus* sp. treated plants. Lowest figures were recorded in control pots. This is very encouraging as all the isolates were isolated from healthy tissue. Sturz (1995) reported that healthy potato tubers (cv. 'Kennebec') were internally colonized by non-pathogenic bacterial populations originating from root zone soil and that the bacteria sampled

Table 5. Evaluation of selected endophytic and rhizospheric bacteria on chickpea plant growth under normal conditions after 30 days

Seed bacterization	Germination (%)	Root length (cm)	Shoot length (cm)	Vigour index
Bacillus megaterium (UASEBCH1)	100	23.67	17.50	4116
Bacillus sp. (UASEBCH2)	100	22.23	16.50	3966
Bacillus circulans (UASEBCH3)	100	21.50	15.00	3650
Erwinia herbicola (UASEBCH4)	100	21.00	16.17	3883
Enterobacter agglomerans (UASEBCH5)	100	21.83	17.83	3716
Pseudomonas fluorescens (PDBCAB2)	100	20.50	16.33	3683
Bacillus subtilis (PDBCN22)	100	21.00	15.17	3677
Control	100	18.67	12.33	3100
LSD (P = 0.05)	-	1.20	0.94	154.19

promoted tuber number and weight. Fu *et al.* (1999) showed that endophytic bacterial strain 73a promoted shoot growth (measured as mean shoot length) by 19.15% in cotton. Rajan *et al.* (2000) reported that endophytic bacteria enhanced tillering, overall growth of the plants and suppressed the pathogens and disease incidence in ginger.

The effect of selected endophytic and rhizospheric bacteria on chickpea plant growth in potted soil pre-inoculated with a soil-borne root rot pathogen *viz.*, *R. solani* was assayed after 14 days of germination (Table 6). The vigour index in all the treatments was significantly higher than the control. The highest index of 831.6 was in *Bacillus* sp. treated and the next best index of 729.4 was in fungicide treated. Involvement of endophytic bacteria in plant growth promotion and disease suppression has been demonstrated (Sturz 1995; Fu *et al.*, 1999; Rajan *et al.*, 2000). Chernin *et al.* (1995) showed that *E. agglomerans* has a complex of chitinolytic enzymes and that the bacteria decreased the incidence of disease caused by *R. solani* in cotton by 64 to 86%. Selected strains of plant growth-promoting rhizobacteria are able to induce a systemic resistance (ISR) in plants. It constitutes an increase in the level of basal resistance to several pathogens simultaneously, which is of benefit under natural conditions where multiple pathogens may be present and when induced plants are infected, disease development or severity are reduced but not prevented. (Loon *et al.*, 1998a and b; Pieterse *et al.*, 2001). We report that endophytic bacteria from healthy plant tissue could play useful role in plant protection.

Table 6. Effect of selected endophytic and rhizospheric bacteria on chickpea plant growth in the presence of *Rhizoctonia solani* after 15 days

Test bacteria	Germination (%)	Root length (cm)	Shoot length (cm)	Vigour index
Bacillus megaterium (UASEBCH1)	61.3 (52.1)	6.5	4.3	600
Bacillus sp. (UASEBCH2)	66.0 (52.8)	6.7	5.9	831
Bacillus circulans (UASEBCH3)	61.6 (51.2)	3.8	4.2	492
Erwinia herbicola (UASEBCH4)	50.1 (46.1)	6.2	4.4	531
Enterobacter agglomerans (UASEBCH5)	59.8 (51.2)	4.0	3.0	418
Pseudomonas fluorescens (PDBCAB2)	61.4 (52.1)	4.8	4.7	583
Bacillus subtilis (PDBCN22)	56.1 (49.6)	3.7	3.5	404
Control	40.8 (39.9)	2.7	2.1	196
Fungicide	75.2 (61.2)	5.2	4.5	729
CD (P = 0.05)	1.98	0.48	0.30	17.03

Changes in phenol content of chickpea plants raised from bacteria treated seeds

Experiments were done to see the changes in phenol content of ten day old seedlings raised from bacteria treated seeds. The bacteria selected were

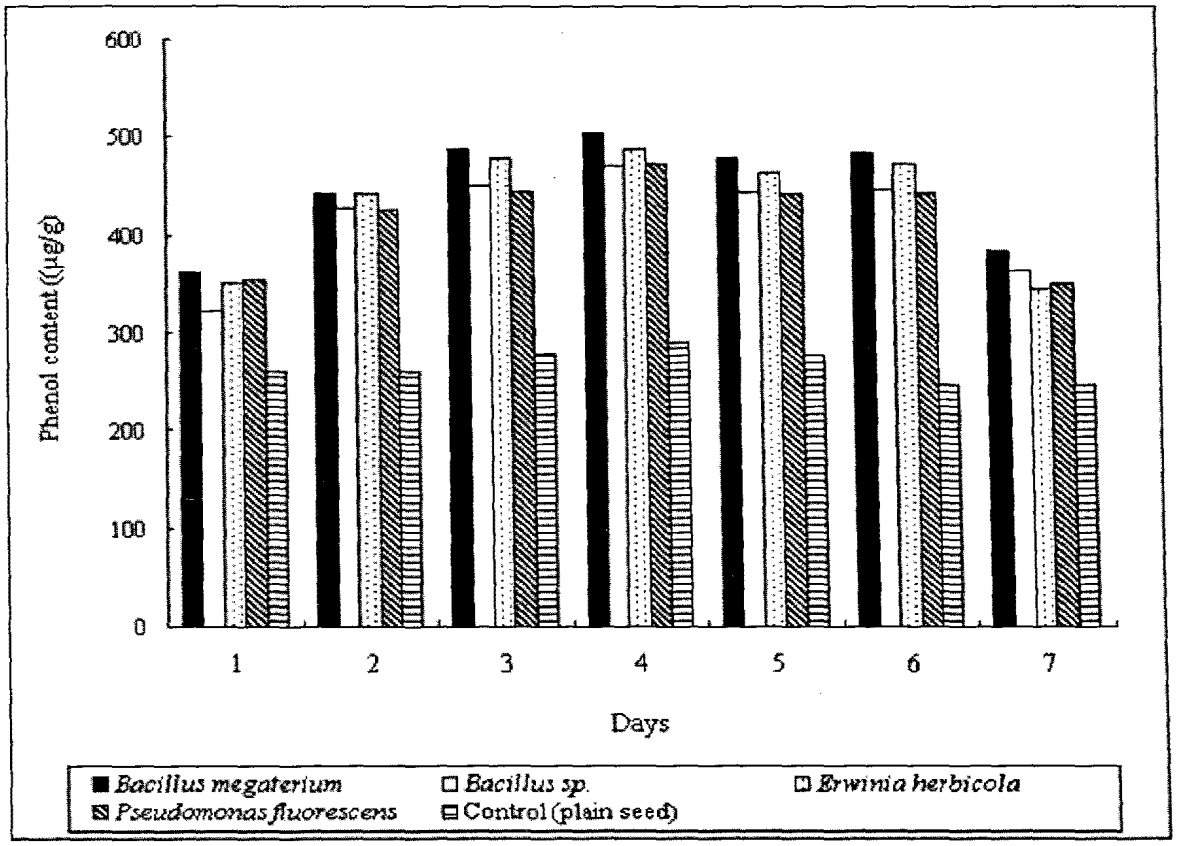

Fig. 1. Changes in phenol content in chickpea plants after seed treatment with rhizospheric and endophytic bacteria

B. megaterium, *Bacillus* sp., *E. herbicola* (endophytes) and *P. fluorescens* (rhizospheric). It was evident from the results (Fig. 1) that significant changes in the phenol content were observed between the treatments.

There was an increase in the phenol content of seedlings for up to day six in all treatments. On day six, maximum phenol content (483.33 µg/g) was noticed in *B. megaterium* treated and the lowest (246.67 µg/g) was in control. There was a decline in the phenol content on day seven in all treatments. Enhanced phenol content in plant tissue after inoculation with test bacteria indicates that the bacteria are involved in induction of systemic resistance (Loon *et al.*, 1998a; Loon *et al.*, 2002; Mauch-Mani, 2002). It was evident from the present study that there were significant changes in the phenol content in the treatments.

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