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Effect of bacterial endophytes isolated from citrus on the physiology of *Brassica Oleracea*

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Abstract

Endophytes have a symbiotic relationship with the different parts of plants and could play a very important role in supporting the plant growth. The effects of endophytic bacterial communities in cabbage have not been studied yet in Pakistan. In present study, ten different genera of bacterial endophytes isolated from citrus leaves were selected to estimate their effects on cabbage physiology at seedlings stage in glass house. To know the extent of colonization of these bacterial endophytes on cabbage as a host or non-host tissues bacterial suspension (10^8 CFU mL^{-1}) were inoculated on the backside of the cabbage seedlings leave by injection syringe in green house. Five weeks after inoculation the plants were analyzed for the physical (SL, RL, SFW, RFW, SDW, RDW), bio-physical (Relative leaf water contents) & physiological (Phenolic, Flavonoids, Total soluble sugars, chlorophyll a, b& carotenoid contents) by using standard methods describe in text. According to results *Bacillus safensis* (shoot length, chlorophyll b contents), *Bacillus megaterium* (root length, phenolic compound), *Pseudomonas aeruginosa* (root fresh weight, total soluble sugars, flavonoids and carotenoids contents), *Staphylococcus haemolyticus* (shoot fresh/dry weight and relative leaf water contents)imparts beneficial effects on physiological functioning of cabbage in comparison to non-inoculated plants. Results indicated that test microbial endophytes possessing a dynamic role to improve plant growth and could be used as inoculants to establish a sustainable crop production system. However, a comprehensive approach is needed to evaluate the potential of these bacterial endophytes to improve the quality and yield in cabbage under field conditions.

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Introduction

Cabbage belongs to family Brassicaceae and it is a good source of mineral and vitamins (Campbell, 2012).Cabbage is grown in Pakistan for edible purpose and it is economically important cash crop of Pakistan (Shah, 2013). It is a cool season crop usually grown in winter and optimum temperature is about 15-20˚C.About 25˚C its growth is stopped.it is mostly grown best in moisture retaining soils and pH range of 6.0-6.5. Pakistan has agriculture based economy so more than half of its population depend on agriculture products. Hence vegetables are most important for the edible crops of Pakistan.

Bacterial endophytes are important plant symbionts that inhabit inside the plant host tissue but do not impose any harmful effect on host. Thee endophytes remain associated with host plants throughout their life cycle (seed germination to fruit development). these microbe can be exist in different parts of plant such as phylloplane (in leaves), rhizosphere (roots), laimospherecaulosp here (in stems), carposphere (in fruits), spermosphere (in seeds), and anthosphere (in flowers) according to (Sessitsch *et al*.,2012). Bacterial endophytes provides biologically active metabolites e.g., bio chemicals, Enzymes, Phytohormones, Nutrients and minerals (Schulz *et al*., 2002). As a result of this interaction plant provides nutrients and shelter for reproduction inside tissue without compromising its own resources (Khan *et al*., 2015). Different strains of bacterial endophytes have been isolated and characterized from other hosts such as Wheat (*Triticumaesitivum*), Tomato (*Lycopersicum esculentum*), Pea (*Pisum sativum*), Corn (*Zea mays*), Canola (*Brassica napus*), (*Avena sativa*), Potato (*Solanum tuberosum*), Barley (*Hordeum vulgare*), Soybean (*Glycine max*), Cucumber (*Cucumis sativa*), Lettuce (*Lactuca serriola*), and Radish (*Raphanus sativus*) (Asaf *et al*., 2017). Some studies have been reported novel bacterial endophytes such as Actinobacter, Arthobacter, Rhizobium, bacillus, Azospirillum, Flavobacterium, Pseudomonas, Enterobacter, and Agrobacterium from other host plants (Gray and Smith, 2005). One of the most important role of bacterial endophytes is in establishment of host plant in a particular harsh environment owing to their role in improvement of plant health and fitness (Achatz *et al*., 2010; Khan *et al*., 2015). The actual mechanism to understand what's going on inside plant tissues after establishment of endophytic bacterial colonization is still un clear but it is believed so these endophytes have some potential to improve the physiological characteristics of plant and help to induce resistant in plant against abiotic and biotic stress. Approximately 300,000 plant species exist on the earth, among them each individual plant is host to one or more endophytes (Strobel *et al.,* 2004). Ultimately there is an opportunity to search new beneficial bacterial endophytes from plants in distinct agro ecological systems. But there are some opportunistic bacterial pathogens which includes *Enterobacter, Burkholderia, Ochrobacterium, Pseudomonas, Staphylococcus, Ralstonia* and *Herbaspirillium* have been identified as colonizer of rhizosphere of plants (Berg *et al*., 2005).Most of the facultative endophytes of rhizosphere from large soil pool have adapted themselves in plants which includes human/animal pathogens as well. Some bacteria that are linked with human infections have been isolated and identified from inside of alfalfa plant (Ponka *et al*., 1995). This area of study needs further investigation to establish the risk if any related to the establishment of endophytic niche for biotechnological applications. In recent era scientist are focusing more on bacteria endophytes in agriculture due to its important role in plant growth promotion as well protection against biotic and abiotic stresses. Formulation of these bacterial endophytes are successfully used in developing world countries. As chemicals are harmful for human health so there is need to produce food commodities with less chemicals so organic farming is best practice now a days. Although it's expensive but scientist are trying to reduce the cost of organic food. The establishment of plant endophyte relationship to modulate plant metabolism are key issues for further research (Chaturvedi, 2016). For successful colonization of endophytes and host endophyte interaction population density of endophytes is highly variable. Mainly it depends on

the genotype of plant and bacterial specie, developmental stage of host, climatic conditions and inoculum density (Pillay and Nowak, 1997; Tan *et al.,* 2003).Therefore, in the present study, isolation and identification of isolated bacterial endophytes were carried out and these strains were inoculated into cabbage plants as well as their effects on plant physiology were examined.

Materials and methods

Leaf samples of different varieties of citrus were collected from the citrus orchids of the Punjab i.e. Lahore, Sargodha, Sahiwal, Mian Chanu, Multan in September, 2015 and preserved in -80 ˚C.

Isolation and identification of bacteria

Isolation of bacterial isolates were carried out by standard isolation method. 3-4 cm section of the leaf were taken and dipped in 1% of Sodium hypochlorite for 3-5 minutes, followed by three consecutive washings with double distilled water (d.dH_2o). The surface-sterilized tissue was crushed in distilled water (100 to 200 µl) for 10 to 20 min. 10 to 20 µl of the suspension was streaked into the nutrient agar plate nutrient agar plate and incubated at 37°C. The purified cultures were identified on the basis of morphology and biochemical process by using Bergey's manual of systematic bacteriology (Garrity, 2005). Strains were submitted into first fungal culture bank of Pakistan (FCBP) and preserved.

Plant growth promoting activities

Cabbage seedling plants were used to test the effects of characterized bacterial strains on the physiological functioning under controlled conditions in glass house. The Cabbage seedlings were planted on sterile (autoclaved) soils in pots having mixture of compost and sandy loam soil.

Inoculum preparation and inoculation

Single celled colony were picked from pure culture and inoculated on test tubes containing 5mL LB-medium were placed in the shaker having temperature 37˚C for overnight then 5mL pre-cultures were transferred into the flasks containing 25 mL LB broth and placed into the shaker for overnight at 37˚C. Pellet was taken by centrifugation of bacterial culture at (6000 rpm for 10 minutes) at 4˚C. The supernatant was discarded and the pallet was dissolved in 15 µl sterilize distilled water and (2% tween 20) was added and left at room temperature for half an hour. 0.1 ml of bacterial suspension (10^8 CFU mL^{-1}) was inoculated into cabbage seedlings by injecting bacterial suspension into the intercellular spaces of leaves with a hypodermic needle into epidermis of the leaf. Randomized complete block design was followed by using three replicates per treatment. Two set of controls were used in this study. Positive control without any treatment, negative control just sterile distilled water injected on the leaves.

After about one month morphometric parameters e.g., shoot/root length (cm), shoot /Root fresh and dry biomass (g), of cabbage seedlings were noted for each treatment followed by three replicates. The bio-physical parameters that were studied include fresh leaves weight (g), dry leaves weight (g), and turgid weight (g), Relative Water Content (%) of the leaves. The leaves were plucked and immediately weighted in weighing balance. Then the leaves were soaked in water for about 8-10 hours to measure the turgid weight of the leaves, after measuring the turgid weight the leaves were placed in oven for drying at 80°C for 24h. Following formula was used to find out The RWC (%) = [(W-DW) / (TW-DW)] × 100; Where W = Sample fresh weight, TW = Sample turgid weight and DW = Sample dry weight.

Biochemical characteristics altered by bacterial inoculums were estimation by following methods. (Arnon, 1949) for Chlorophyll contents, (Malik and Srivastava, 1985) for total soluble sugars, (Bates *et al.*, 1973) for Proline contents, (Kaur *et al.*, 2002) for the total phenolic content, (Chang *et al.*, 2002) for the total flavonoid content , (Lowry *et al.*, 1951) for Protein content.

Statistical analysis

All the collected data were subjected to statistical analysis by using one factorial randomized complete block design with three replicates. Analyses of variances were carried out and means were separated by least significant difference test (LSD).

All the data was statistically analyzed at 5% level of probability. The entire statistical work was done by using the computer package Statistics 8.1.

Results and discussion

This study provides information about the characterization of bacterial species from different varieties of citrus as well as their effects on physical and physiological functioning of cabbage seedlings. Ten Bacterial isolates were characterized based on morphology and biochemical test following Bergey's manual of systematic bacteriology (unpublished).

Table 1. Shoot length, root length(cm),RL/SL ratio, Shoot /root fresh and dry biomass as well as leaf relative water contents of Cabbage (*Brassica oleracea)* seedlings grown under greenhouse conditions inoculated with bacterial endophytes.

Treatments	Shoot length (cm)	Root Length (cm)	Root fresh weight (g)	Shoot fresh weight (g)	Root dry weight (g)	Shoot dry weight (g)	Relative leaf water content (%)
Control positive	9.033abc	3.666a	0.104g	1.81g	0.057ab	0.153h	76.27ab
Control negative	9abc	2.366a	0.178cd	2.07f	0.0426ab	0.183g	52.09de
Bacillus safensis	12.166a	7a	0.143f	2.96c	0.113a	0.227f	38.52fg
Pseudomonas sp.	7.266bc	4a	0.256a	1.523i	0.047ab	0.135i	43.88ef
Enterococcus faecalis	8.66abc	5.333a	0.15ef	2.953c	0.054ab	0.22f	30.99g
Bacillus megaterium	8.933abc	3a	0.145f	1.723h	0.034ab	0.22f	53.50de
Pseudomonas aeruginosa	10.066ab	5.833a	0.193cd	3.156b	0.052ab	0.273d	69.18bc
Brevibacillus borstelensis	10.667ab	4.4a	0.066h	2.826d	0.0126b	0.3c	54.25de
Staphylococcus haemolyticus	9.7ab	6.9a	0.196c	4.04a	0.042ab	0.372a	179.39a
Enterobacter hormachei	10.733ab	7a	0.233b	3.153b	0.066ab	0.346b	60.98cd
Bacillus cereus	10.333ab	5.433a	0.135b	2.966e	0.0316ab	0.253e	57.30d
Proteus mirabilis	9abc	5.666a	0.170de	2.293e	0.0433ab	0.123i	76.43b

All the identified strains were belongs to class Firmicutes (*Staphyloccoccus haemolyticus; Enterococcus faecalis; Bacillus safensis; Bacillus megaterium; Bacillus cereus; Brevibacillus borstelensis)* and member of class Proteobacteria *(Pseudomonas sp.; Pseudomonas aeruginosa; Enterobacter hormachei; Proteus mirabilis*).

Diagrammatic representation of the test strains used in this study and symptoms appears after inoculation is shown in (Fig.1).

In vitro screening of isolated bacterial isolates for plant growth promoting potential in Cabbage

Effects of different endophytic bacterial species were studied on cabbage(*Brassica Oleracea*) to check the host range of the isolated bacterial strain or to check either these bacterial isolates have significant effect on growth of cabbage or not. After inoculation different morphometric parameters were recorded after five weeks e.g. shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight. Bio-physical analysis relative water content was also performed to detect the effect of these endophytic bacteria on host plants physiology and also to check either these bacterial species reduce or enhance the plant growth. Cabbage seedling was inoculated by injected the bacterial cell suspension into back side of leaves (Table 1).

Bacillus and pseudomonas sp. have been reported to increase shoot/ root length or plant growth promotion in Tomato, Maize, Rice, Grape wine, Sugarcane and Sugar beet Through various mechanisms (Mehnaz, 2011; Mirza *et al*., 2006; Wang *et al*., 2009). In present studies similar trend was found related to increase of vegetative growth of plant by inoculation of endophytes.

Table 2. Flavonoids, Phenolics, Total soluble sugars, Chlorophyll a, Chlorophyll b, Carotenoids of Cabbage (*Brassica oleracea)* seedlings grown under greenhouse conditions inoculated with bacterial endophytes.

Treatments	Flavonoids	Phenolic	Total soluble sugars	chlorophyll a	Chlorophyll b	Carotenoids
Control positive	17.44 abc	1.612 a	5.22bc	0.0206 a	22.3 b	6.097 a
Control negative	13.48 cde	1.29 bc	9.84a	0.081 a	27.93 a	5.49 a
Bacillus safensis	11.68 de	0.95 d	5.54 b	0.061 a	29.13 a	5.74 a
Pseudomonas sp.	20.53 a	1.60 a	3.62 cd	0.087 a	17.033 c	9.57 a
Enterococcus faecalis	15.66 bcd	1.55 a	2.54 de	0.105 a	22.76 b	6.18 a
Bacillus megaterium	18.941 ab	1.61 a	2.52 de	0.103 a	21.69 b	3.58 a
Pseudomonas aeruginosa	10.11 e	1.53 a	0.58f	0.170 a	28.77 a	5.13 a
Brevibacillus borstelensis	15.27 bcd	1.29 bc	0.37f	0.034 a	21.54 b	3.77 a
Staphylococcus haemolyticus	12.52 de	1.27 bc	0.303f	0.055 a	15.30 c	2.24 a
Enterobacter hormachei	14.53 bcde	1.24 c	4.95bc	0.0605 a	20.25 b	4.89a
Bacillus cereus	16.44 abcd	1.49 ab	5.28bc	0.025a	21.56 b	4.94a
Proteus mirabilis	14.89 bcde	1.11 cd	0.95ef	0.052 a	21.5 b	3.03 a

The shoot/root length of cabbage seedling were showed statistically non- significant results for allthe isolates as compared to control ($p<0.05$) while the remaining isolates have no significant effect on the seedlings shoot length as compared to control. Maximum shoot length (12.16 cm) were observed in *Bacillus safensis* while minimum (7.26 cm) in *Pseudomonas sp.* as compared to positive control (9.03 cm); negative control (5.5cm) respectively. Maximum root length (7 cm) were observed in *Bacillus safensis* while minimum (3cm) in *Bacillus megaterium* as compared to positive control (3.66 cm); negative control (2.36cm) respectively.

The shoot fresh and dry weight and root fresh weight of cabbage seedling were showed statistically significant results except root dry weight that showed non-significant results for allthe isolates as compared to control ($p<0.05$). Maximum shoot fresh weight (4.04 g) were observed in *Staphylococcus haemolyticus* while minimum (1.523g) in *Pseudomonas sp.* as compared to positive control (1.81g); negative control (2.07g) respectively. Maximum shoot dry weight (0.372 g) were observed in *Staphylococcus haemolyticus* while minimum (0.123 g) in *Proteus mirabilis* as compared to positive control (0.153g); negative control (0.183g). Maximum root fresh weight (0.256 g) were observed in *Pseudomonas sp.* while minimum (0.066g) in *Brevibacillus borstelensis* as compared to positive control (0.104g); negative control (0.178g) respectively. Maximum root fresh weight (0.52 g) were observed in *Pseudomonas sp.* while minimum (0.112 g) in *Enterococcus faecalis* as compared to positive control (0.1366g); negative control (0.45g).

Relative water contents RWC demonstrate the genetic capability of the crop plants to combat or tolerate under water limited conditions where inoculation can be helpful. Leaf relative water contents of cabbage seedling were showed statistically significant results for allthe isolates as compared to control ($p<0.05$) while the remaining isolates have no significant effect on the seedlings leaf relative water contents(%) as compared to control. Maximum leaf relative water contents (179.39 %) were observed in *Staphylococcus haemolyticus* while minimum (30.99%) in Enterococcus faecalis as compared to positive control (76.27%); negative control (52.099%).

Similar studies on the inoculation of the bacterial endophytes and their effect on relative water contents of wheat has been conducted by Vardharajula *et al.* (2011) and Sandhya *et al.* (2010), hence the bacterial inoculation reduced the membrane damage in drought stressed plants compared to control.

Fig.1. Pictorial representation of the Cabbage seedlings infected with bacterial inoculums by injecting on the back side of leaves A) Positive Control B)Negative control C)*Staphylococcus haemolyticus* D) *Proteus mirabilis* E) *Enterobacter hormachei* F) *Bacillus safensis*G) *Bacillus cereus* H) *Brevibacillus borstelensis* I) *Bacillus megaterium* J) *Pseudomonas sp.* K) *Pseudomonas aeruginosa* L) *Enterococcus faecalis*.

Physiological parameters of cabbage seedlings

The phenolic contents (mg of QE/g) of cabbage seedling were showed statistically significant results for allthe isolates as compared to control ($p<0.05$) while the remaining isolates have no significant effect on the seedlings Phenolic contents (mg GAE/g) as compared to control. Maximum Phenolic contents (1.61 mg of QE/g) were observed in *Bacillus megaterium* while minimum (0.952 mg of QE/g) in *Bacillus safensis* as compared to positive control (1.61 mg of QE/g); negative control (1.29 mg of QE//g) (Table 2). Antioxidants have the ability to create immunity in plants against various biotic and abiotic stresses such as phenolic and flavonoid contents. The current results are in agreement with previous studies reported by (Swarnalatha *et al*., 2015).

The flavonoid contents (mg of QE/g) of cabbage seedling were showed statistically significant results for allthe isolates as compared to control ($p<0.05$) while the remaining isolates have no significant effect on the seedlings Flavonoid contents (mg(QE)/g) as compared to control. Maximum flavonoid contents (20.53 mg (QE)/g) were observed in *Pseudomonas sp*. while minimum (10.11 mg of QE/g) in *Pseudomonas aeruginosa* as compared to positive control (17.44 mg of QE/g); negative control (13.48 mg of QE/g). Flavonoids have important role in the stabilization of lipids oxidation process in plants and it is an antioxidant as itself (Kostova *et al*., 2011).this study relates with the results of the (Jalgaonwala *et al.,* 2011) about the increase of flavonoids by Serratia sp. associated with host plant *Pongamia glabra*.

Whenever plant confronted with any stress accumulation of carbohydrates and sugars starts in plant tissue as it increase the cellulolytic and

pectolytic enzymes activity which are important for pathogenesis. There is association between sugars level and pathogen development if there is low level of sugars mean pathogen has utilized maximum sugars and growing inside plant tissues hence as a result infection of plant occurs, but with high sugar level chances of infection will be less (Biale, 1964; Kadioglu and Yavru, 1998). Hence total soluble sugars play an important role in disease resistance because sugars are precursor for the production of phytoalexins and phenolic which suppress/ reduce the pectolytic and cellulolytic enzyme that are crucial one for pathogenesis. Nath *et al.* (2015) reported that the Total soluble sugars reduced in inoculated plants as compared to non-inoculated one which opposed the results of current study in which increase of TSS were found in inoculated plants. The total soluble sugars (mg/g) of cabbage seedling were showed statistically significant results for all the isolates as compared to control ($p<0.05$) while the remaining isolates have no significant effect on the seedlings total soluble sugars (mg/g) as compared to control. Maximum total soluble sugars (0.58 mg/g) were observed in *Pseudomonas aeruginosa* while minimum (0.095mg/g) in *Proteus mirabilis* as compared to positive control (0.522 mg/g); negative control (0.984 mg/g).

The chlorophyll a contents (mg/g) of cabbage seedling were showed statistically non-significant results for all the isolates as compared to control ($p<0.05$) while the remaining isolates have no significant effect on the seedlings chlorophyll a contents (mg/g) as compared to control. Maximum chlorophyll a contents (0.137mg/g) were observed in *Brevibacillus borstelensis* while minimum (0.010 8mg/g) in *Bacillus cereus* as compared to positive control (0.020mg/g); negative control (0.08 mg/g). The chlorophyll b contents (mg/g) of cabbage seedling were showed statistically significant results for all the isolates as compared to control ($p<0.05$) while the remaining isolates have no significant effect on the seedlings chlorophyll b contents (mg/g) as compared to control. Maximum chlorophyll b contents (29.13mg/g) were observed in *Bacillus safensis* while minimum (9.936mg/g) in *Pseudomonas aeruginosa* as compared to positive control (28.3mg/g); negative control (27.93mg/g).

The carotenoid contents (mg/g) of cabbage seedling were showed statistically non-significant results for allthe isolates as compared to control ($p<0.05$) while the remaining isolates have no significant effect on the seedlings carotenoid contents (mg/g) as compared to control. Maximum carotenoid contents (9.57mg/g) were observed in *Pseudomonas sp*. while minimum (2.31mg/g) in *Staphylococcus haemolyticus* as compared to positive control (6.0497mg/g); negative control (5.492mg/g). Bacterial endophytes are most frequently involved in the activation of physiological alterations that promote the growth and development of plants (Van Overbeek, 2008).

Our investigation relates to study of Padder *et al.* (2015) for the improvement of chlorophyll a, b and carotenoids contents as well as other growth promoting physiological parameter after inoculation of *Pseudomonas sp*. Positive beneficial effects of bacterial inoculants in sugarcane plant under field condition has been reported by Chauhan *et al.* (2013) after six months plant showed improved chlorophyll, nitrogen contents as well as yield. Increase of chlorophyll contents through bacterial inoculum could be due to increase of electron transport rate, opening of stomata as well as chlorophyll metabolism.

This could be due to production of unknown compounds by bacterial endophytes, ultimately increase of photosynthesis happens which is important plant growth parameter (Shi *et al.*, 2010). Also these bacteria upgrade the photosynthetic genes related to Ferredoxin and NADPH ferredoxin in leaves (Bilgin *et al.,* 2010). It has been reported that *pseudomonas* sp. inoculation in wheat increase grain yield and nutrients (N, P, K, Ca) uptake from soil (Hassan and Bano, 2015).Another report that beneficial bacterial endophytes increase plant growth in tomato (Ardebili *et al.,* 2011) and Kang *et al.*

(2007) reported the positive effect of pseudomonas sp. on yield and nutrient uptake of pepper.

Conclusion

Two strains *Bacillus safensis* (shoot length, chlorophyll b contents) and *Bacillus megaterium* (root length, phenolic compound) give best results while *Pseudomonas aeruginosa*, *Pseudomonas sp.* enhance root fresh weight, total soluble sugars, flavonoids and carotenoids contents. *Staphylococcus haemolyticus* gives maximum shoot fresh/dry weight and relative leaf water contents in comparison to non-inoculated plants. After inoculation of plants its seems more healthy than non-inoculated ones and showed better growth trend so it might be assumed that cabbage is good host for the tested bacterial endophytes, but this behavior will vary according to genotype of crop plants and environmental condition. Successful colonization of endophytes inside plant tissue are very important aspect to study the host endophyte interaction so according to results these bacterial endophytes can be test in field conditions on cabbage or other vegetable host in order to ensure the results. These growth promoting bacterial strains could be used as an alternate to fertilizers for sustainable crop production.

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