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Antioxidant producing endophytic bacterial consortium as biological tool for enhancing the antioxidant activity of bhendi under salt stress

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Abstract
The crops exposed to salinity face reduced crop growth and productivity due to intervention in the physiological activities like ion toxicity, nutrient deficiency and oxidative stress. Therefore, it is critical that the plant counteract the production of reactive oxygen species with mechanisms for neutralizing them. Antioxidant enzymes are essential components of the plant's antioxidant defence system. The antioxidant enzyme activity and role in scavenging oxidative stress of certain endophytic microbes may be useful as growth promoting microbial agent for enhancing the vigour and tolerance of agricultural and horticultural crops under salt stress. In the present study two experiments have been conducted to standardise the method of application (Foliar spray, Soil Application, Foliar spray +Soil application) and to find the impact of microbial consortium containing antioxidant producing bacterial endophytes on growth and antioxidant activity of bhendi (CoBh H1) under *in vitro* condition by implementing four treatments as Control, Biofertilizer + Biocontrol agents, AOE Consortium, Biofertilizer + Biocontrol agents + AOE Consortium. Among the various methods of application tried foliar spray performed better by recording higher Plant height (19.82 cm) and stem girth (1.8cm) on 60th DAS and leaf Area of (32.18cm^2, 101.53cm^2, 77.37cm^2) on 30,45 and 60 DAS. In the second experiment, plants treated with AOX consortium recorded higher plant height (19.2 cm), stem girth (1.9cm), average leaf number (10.3) and catalase (145 U mg protein^{-1}) & peroxidase activity (21.6 U mg protein^{-1}) on 60 DAS which were statistically on par with Biofertilizers + biocontrol agents imposed plants.

Keywords: Salt stress, bhendi, endophytic consortium, AOX, antioxidants, peroxidase, catalase

Introduction
Salt tolerance of plants is complex due to interaction of many molecular, biochemical and physiological phenomenon involved and these factors accelerate the reactive oxygen species scavenging systems (Zhu, 2003) [1]. Non enzymatic antioxidant compounds such as phenolic compounds, ascorbic acid, tocopherols, glutathione and carotenoids are employed by plants to eliminate ROS. Apart from that, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (POX) scavenge ROS and are essential components of the plant's antioxidant defence system. Moreover, the ability of certain plant species to increase production of antioxidant compounds and enzymes in response to salinity has been correlated with salt tolerance (Lopez *et al.*, 1996; Shalata *et al.*, 2001) [2, 3]. The exogenous application of proline can also protect cell membranes from salt induced oxidative stress by enhancing activities of various antioxidants (Yan *et al.*, 2011) [4] wherein the plants have to spend more energy on antioxidant enzyme production. Supplementing through biological agents especially endophytes would have a greater impact on plant health than those active in the rhizosphere since they are protected within plant tissues. In this study an attempt was made to isolate bacterial endophytes from vegetable crops grown in saline soils and thier antioxidant production ability was determined. An AOX consortium was prepared from the screened endophytes and the method of application of this consortium to bhendi crop was standardised. In another study the impact of this AOX consortium on growth and antioxidant activity of bhendi was studied under salt stress condition.

Materials and Methods
Isolation of endophytic bacteria
In this study bacterial endophytes were isolated from stem tissues, leaves or roots of Rice, Maize, Bhendi, Sorghum, cumbu and Cotton by sterilizing the roots by sequential immersion in 70% (v/v) ethanol for 5 min, and 1% sodium hypochlorite solution for 20 min and rinsed four times in 0.02 M sterile potassium phosphate buffer (PPB, pH 7.0). The samples were then

washed in sterile distilled water for three times to remove surface sterilization agents. The samples were soaked in 10% (w/v) NaHCO3 solution for 10 min to retard the growth of endophytic fungi. Each sample (0.5 g) was homogenized in sterile pestle and mortar using 9.5 mL of the buffer. Serial dilutions of the homogenate up to (10^{-8}) were made in PPB. Dilutions of all the samples were plated on tryptic soy agar. The plates were incubated at 28 ºC for 48-72 hrs. All the isolates were screened for the production of catalase (CAT), peroxidise (POX), Super Oxide Dismutase (SOD) and phenolic compounds.

Preparation of microbial consortium

Microbial consortia are a group of different species of microorganisms that act together as a community. The best performimg 10 isolates were selected and their compatibility was tested by cross streak assay. The compatible bacterial endophytes were identified in MALDITOF and grown in 100ml of nutrient broth kept in Incubator shaker at 180 rpm at 30 ^{0}C and the 24 hrs old culture was used for the treatments in pot culture experiment.

Standardization of method of application of AOX consortium

The pot culture experiment was carried out at Anbil Dharmalingam Agriculture College and Research Institute, Navalur kuttapattu, Trichy using soil collected from Manikandam Block with an EC of 6. The crop used was Bhendi -Syngenta597 (F1 Hybrid) with 4 treatments and 5 replications *viz.*, C-control, T1-Soil application (after germination, 10DAS, 25 DAS, 40DAS), T2- Foliar spray (10 DAS, 25 DAS, 40 DAS), T3-Soil application + Foliar spray (after germination, 10 DAS, 25 DAS, 40DAS). Plant height, stem girth and leaf area were measured at 30, 45 and 60 DAS and results were tabulated. Leaf area was calculated from the product of length and breadth and expressed in cm^2.

Impact of AOX consortium on growth and enzymatic antioxidants of Bhendi crop

The best performing method of application was selected for studying the impact of application of AOX consortia on the growth and enzymatic antioxidant production of Bhendi crop. Bhendi crop (CoBh H1) was raised in pots by implementing four treatments as follows: T1 – Control, T2 – Biofertilizer + Biocontrol agents, T3 – AOE Consortium, T4 - Biofertilizer + Biocontrol agents + AOE Consortium. The plant height, stem girth, Leaf number, catalase and peroxidase activity were estimated on 30, 45 and 60 DAS.

Peroxidase (POD) POD (EC 1.11.1.7) activity was determined by the method of Chander (1990). The assay mixture (3 ml) was prepared using 50 mM potassium phosphate buffer (pH 7.0), 0.2mL of o-phenylenediamine and 30 µl of leaf enzyme extract. By adding hydrogen peroxide (H_2O_2) the reaction was initiated. The peroxidase activity was estimated by measuring the H2O2 dependent oxidation of O-phenylenediamine at 450 nm and expressed in units per mg of protein in comparison with the standard enzyme.

Catalase (CAT) CAT (EC 1.11.1.6) activity was determined by the protocol of Masia (1998). The assay mixture (3.0 mL) included 50 mM potassium phosphate buffer (pH 7.0), 0.3mL of hydrogen peroxide (H2O2) and 100 µL of the leaf enzyme extract. The reduction of absorbance at 240 nm was estimated for finding the reduction in H_2O_2 and expressed in units per mg of protein.

Result and Discussion

In many research works, endophytic bacteria have been isolated from many different plants including trees (Pine, Yew), fodders (alfalfa, sorghum, clover), vegetables (carrot, radish, tomatoes, sweet potatoes, lettuce, soybean), fruits (banana, pineapple, citrus), cereal grains (maize, rice, wheat) and other crops (sugarcane, marigold, coffee) (Rosenblueth and Martinez-Romero 2006) [5]. In this present study the bacterial endophytes used for this study were isolated from crops like Rice, Cotton, Maize, Cumbu, Sorghum, and Brinjal. Totally 35 endophytes were isolated ; 6 bacterial endophytes from rice, 7 from cotton, 10 from Maize, 6 from Sorghum, 3 from Cumbu and 3 from Bhend cropi. After screening for their antioxidant activity of CAT, POX, SOD, phenols (data not shown) the best performimg 10 isolates were subjected to compatibility studies and only 4 were found to be compatible (R3, Ma5, So2, Cu3). The isolates were identified as *Bacillus megatherium* (R3), *Bacillus megatherium* (Ma5), *Bacillus cereus* (So2) and *Pseudomonas aeuriginosa* (Cu3) by running the samples in MALDI-TOF (Bruker-Microflex).

Usage of biological agents as consortium is proved to be highly efficient rather than using them as single culture. The mycoparasite *Trichoderma harzianum* inhibits *Piriformospora indica* growth *in vitro* and root colonization but inoculation of pepper plants with *P. indica* and subsequently with *T. harzianum* resulted in higher plant dry weights compared to single inoculations (Anith *et al.*, 2011) [6]. Hence the consortium of the 4 screened endophytic bacteria was prepared and used for inoculation studies.

Normally endophytes promote plant growth by a number of mechanisms, which include phosphate solubilisation activity (Verma *et al*., 2001; Wakelin *et al*., 2004) [7, 8], indole acetic acid (Lee *et al.*, 2004) [9] and the production of siderophore (Costa and Loper, 1994) [10]. It is also proved that endophytic organisms can also supply essential vitamins to plants (Pirttila *et al.*, 2004) [11]. Moreover a number of other beneficial effects on plant growth have been attributed to endophytes and include osmotic adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals and alteration of nitrogen accumulation and metabolism (Com-Pant *et al*., 2005) [12]. In the present study also the results were in accordance with the previous findings regarding the role of endophytes in plant growth.

Plant height (13.47, 17.48, 19.82 cm) was found to be statistically significant in leaf spray (T2) treatment on 30,45 and 60 DAS than control and other treatments followed by T3 treatment (11.55, 13.09, 17.15 cm). T1 was statistically on par with control on 45 and 60 DAS. However, statistically on par with soil application +leaf spray (17.15cm) treated plants. But soil application (T1) recorded lower value (12.03cm) than control (13.5 cm). Stem diameter was more in foliar spray treatment (1.83cm) followed by soil application (1.65cm) on 60 DAS and T1 (1.65 cm) & T3 (1.59 cm) were statistically on par. But we observed low value of stem diameter in T3 treatment than control and other treatments.

Table 1: Plant height and stem girth of Bhendi crop under pot culture experiment

	Plant Height (cm)			Stem Girth (cm)		
Treatments	**30 DAS (cm)**	**45 DAS (cm)**	**60 DAS (cm)**	**30 DAS (cm)**	**45 DAS (cm)**	**60 DAS (cm)**
Control (C)	8.13^{d}	12.81^{bc}	13.53^{c}	0.64^{c}	1.09^{b}	1.44^{c}
Soil application (T1)	9.62 ^{c}	12.96 ^{bc}	13.03^{c}	0.80^{b}	1.26^{a}	1.65^{bc}
Foliar spray (T2)	13.47 ^{a}	17.48^{a}	19.82^{a}	0.93^{a}	1.33^{a}	1.83^{a}
Soil application +Foliar spray (T3)	11.55 ^{b}	13.09^{b}	17.15^{b}	0.86^{ab}	1.22^{a}	1.59^{bc}

Different letters in a single column show statistically significant differences for $P<0.05$

Foliar spray (T2) treatment also recorded higher leaf area (32.18 cm^2, 101.53cm^2, 77.37cm^2) followed by T3 on 30, 45 and 60 DAS. Soil application (T1) treatment registered lower leaf area than other two treatments but was higher than control. However on 60 DAS (23.75cm^2) it recorded lower value than control. In general, microbial endophytes enter plant tissues mainly through the root zone; however, aerial plant parts like flowers, stems and cotyledons may also be used for entry (Kobayashi and Palumbo, 2000) [13] and also enter via germinating radicles (Gagne *et al.*, 1987) [14], secondary roots (Agarwal and Shende, 1987) [15], stomata (Roos and Hattingh, 1983) [16] and foliar damage (Leben *et al.*, 1968) [17]. In the present study the superior performance of foliar spray showed that these endophytes prefer the entry through stomata and foliar damage rather than root entry.

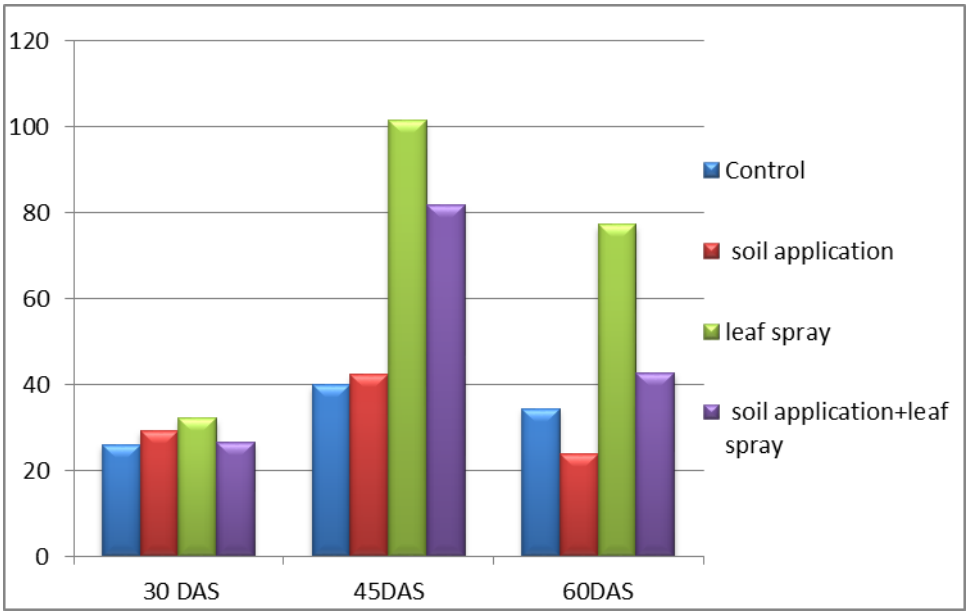

Fig 1: Leaf Area (cm^2) of Bhendi crop under pot culture experiment

In the second pot culture experiment conducted to study the impact of AOX consortia on growth and enzymatic antioxidants of Bhendi crop, the foliar spraying of AOE consortium (T3) recorded higher plant height (19.2 cm), stem girth (1.9cm), average leaf number (10.3) and catalase (145 U mg $protein^{-1}$) & peroxidase activity (21.6 U mg $protein^{-1}$) on 60 DAS which were statistically on par with 'Biofertilizers + biocontrol agents' (T2) imposed plants.

Table 2: Application of AOX consortia on growth and enzymatic antioxidant production of Bhendi crop on 60 DAS

Treatments	**Plant Height (cm)**	**Stem girth (cm)**	**Leaf Number**	**Catalase Activity Units/mg protein $^{-1}$**	**Peroxidase Activity Units/mg protein $^{-1}$**
T1 – Control	11.4±0.82	1.6±0.035	8.1±0.91	96.5±7.02	12.05±1.05
T2 – Biofertilizer + Biocontrol agents	18.3±0.63	1.8±0.034	10.2±0.95	139.6±6.12	20.1±1.23
T3 – AOE Consortium	19.2±0.61	1.9±0.028	10.3±0.75	145.3±8.37	21.6±0.98
T4 - Biofertilizer + Biocontrol agents + AOE Consortium	17.5±0.55	1.6±0.041	9.6±0.84	110.2±8.06	17.34±0.96

Values are mean ± SD; sample (n) = 5

CD 0.91 0.1 0.45 7.64 1.9

It is observed that an endophytic *Phomopsis sp* could promote growth, photosynthesis and antioxidant activity in rice which is a non host plant (Yuan *et al.*, 2007) [18]. Pestacin and isopestacin obtained from endophytic *Pestalotiopsis microspora* displayed antimicrobial and antioxidant activity (Strobel *et al.*, 2002) [19]. Under water stressed conditions tomato plants inoculated with *Enterobacter* P-68, *Enterobacter* P-46, *Enterobacter* P-39 and *Bacillus* G-4 recorded the highest activities of peroxidase, superoxide dismutase, catalase and glutathione reductase respectively (Hema Bindhu *et al.*, 2018) [20].

Gusain *et al.*, (2015) [21] have reported that the inoculation of plant growth promoting consortium was found to enhance the plant growth and induction of SOD, CAT, peroxidase (POD),

APX and lower the level of H_2O_2, malondialdehyde (MDA) in rice (*O. sativa*) under drought stress conditions compared to control. In the present study it was obvious that the AOX consortium could enhance the antioxidant activity of bhendi under salinity condition of EC 6.0. Similarly it has also been shown that the inoculation of plant growth promoting bacteria has been shown to significantly elevate of the anti-oxidant enzyme levels of lettuce plants exposed to high levels of salinity (Han and Lee 2005) [22]. These results highlight that the antioxidant producing endophytic bacterial consortium could be effectively used for the modulation of growth and antioxidant system of bhendi crop exposed to salt stress conditions.

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