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POSITIVE ROLE OF "INTERGENERIC MICROBIAL CO-AGGREGATES", COMPRISING OF *PSEUDOMONAS* AND *PAENIBACILLUS* CELLS, ON THE ENHANCEMENT OF INDUCED SYSTEMIC RESISTANCE (ISR) IN MAIZE – *HELMINTHOSPORIUM TURCICUM* PATHOSYSTEM UNDER SEMIARID CONDITION

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ABSTRACT

The application effect of different formulations of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* cells *viz*., single strain inoculation, co-inoculation and biofloc application together with challenge inoculation of *Helminthosporium turcicum* on the enhancement of growth and yield parameters in maize with special emphasis to Induced systemic resistance (ISR) mediated biocontrol against leaf blight disease was studied under pot culture condition with maize cv. Co 1. The application of *P. fluorescens* and *P. polymyxa* cells, as biofloc, augmented the growth and yield parameters *viz*., plant height, root and shoot dry weight, phosphorous, carbohydrate, chlorophyll and IAA content, grain, stalk and cob yield of maize cv. Co 1 and altered the biochemical and physiological parameters of maize plant to a maximum level followed by co-inoculation and single strain application. Moreover, the application of *P. fluorescens* and *P. polymyxa* cells, as biofloc, increased the total phenol content and defense enzymes content of maize, reduced the reducing and non-reducing sugar content to a higher level that eventually lead to the reduction in *H. turcicum* incidence in maize. The results of the present study clearly revealed the positive role of biofloc formulation of *P. fluorescens* and *P. polymyxa* cells on the enhancement of maize productivity and maximization of ISR mediated biocontrol in maize – *H. turcicum* pathosystem under semiarid condition together with a reduction in 25 per cent 'P' fertilizer application.

KEYWORDS:Maize, Intergeneric microbial coaggregates, *Pseudomonas fluorescens*, *Paenibacillus polymyxa*, growth stimulation, Induced systemic resistance (ISR)

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INTRODUCTION

Maize (*Zea mays* L.) is the third major crop of the world after wheat and rice which provides more nutrients for humans and animals than any other cereals and the same is grown across a wide range of agro-ecological zones including semiarid condition. Globally, India is the fifth largest producer of maize where the same is mainly grown under semiarid condition. Numerous biotic and abiotic factors may limit the productivity, of that low soil fertility and incidence of diseases are considered to be the major constraints. Phosphorous is generally deficient in semiarid soils and the same is fixed as water insoluble calcium phosphate. Fixation of 'P' in this soil eventually lead to the reduction in BNF (biological nitrogen fixation) and the availability of other nutrients[1]. Moreover, the leaf blight disease of maize (*Helminthosporium turcicum*), one of the ubiquitous and most destructive fungal disease of maize and causing an yield loss up to 90 per cent. Hence, the productivity of maize (yield/ha) must be greatly enhanced by providing additional nutrient inputs and through effective control of phytopathogens. Now-a-days, maize production management strategies in semiarid region mainly focus on chemical amelioration, including, the use of synthetic chemical fertilizers and pesticides at high rates to enhance the per hectare yield of crop[2]. The enormous use of synthetic chemicals are too expensive and also leads to environmental hazards. In this context, plant growth promotion by free living, beneficial soil microorganisms, as a biological approach, might be an alternative strategy to overcome the biological and environmental hazards posed by the persistent use of synthetic chemicals[3,4] and application of biocontrol agents for crop protection is very significant as it has several advantages such as possibility of multiple pathogen suppression, low cost and promotion of soil fertility[5]. *Pseudomonas fluorescens* and *Paenibacillus polymyxa* are two important PGPR which are frequently encountered from the rhizosphere of maize, grown under semiarid condition. *P. fluorescens* has emerged as the biggest, potentially the most promising group among fluorescent Pseudomonads, involved in plant growth stimulation and biocontrol of plant diseases of many crop plants due to the production of secondary metabolites, such as, siderophores, antibiotics and phytohormones[6,7]. *P polymyxa* (*Bacillus polymyxa*)[8], a common soil bacterium which possess a wide range of activities, including, plant growth promotion and biodissolution of plant nutrients, including phosphorous in the rhizosphere of crop plants[9,10]. Agricultural bioinoculant formulation plays a crucial role on the inoculation processes which determine the potential success of the bioinoculants. In the recent years, several new agricultural bioinocula formulations have been proposed, of which EPS mediated "Intergeneric microbial coaggregates" seems to be a promising one for the production of multipurpose agricultural bioinoculant with multiple benefits[11]. However, there were no earlier reports regarding the development and use of "Intergeneric microbial coaggregates" in maize crop, available. Hence, the present research work has been undertaken with an aim to exploit the positive role of "Intergeneric microbial coaggregates", comprising the genera of *Pseudomonas* and *Paenibacillus*, on plant growth stimulation, 'P' solubilization and ISR mediated biocontrol against *Helminthosporium turcicum* in maize crop grown under semiarid condition.

MATERIALS AND METHODS

Culture conditions

The efficient *Pseudomonas fluorescens* and *Paenibacillus polymyxa* isolate *viz*., PF-16 and Pb-16, isolated from the rhizosphere of maize, grown at Kadampuliyur, Chidambaram taluk, Cuddalore district, Tamil Nadu state, India were used in the present study. The *P. fluorescens* and *P. polymyxa* isolates were maintained in King's medium B[12] and Nutrient Glucose agar[13] medium, respectively, and incubated at 37ºC ± 2ºC, with monthly transfer. *H. turcicum* (AU-1),

obtained from Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Annamalai Nagar, India, was used as a reference strain for the biocontrol study and the same was maintained in Potato Dextrose agar (PDA) slants and examined periodically for its virulence.

DIFFERENT FORMULATIONS OF PSEUDOMONAS AND PAENIBACILLUS CELLS

(i) Single strain inoculation

Pseudomonas fluorescens (PF-16) and *Paenibacillus polymyxa* (Pb-16) isolates were grown separately in King's medium B and Nutrient Glucose medium duly supplemented with 0.05% yeast extract (W/V) in a shaking bath at 30ºC ± 2ºC for 24 h. Then, the medium was centrifuged at 5000 × g for 10 min to harvest the log phase cells and the pellets were washed three times with 0.1 M phosphate buffer (pH 6.8). Finally, the cells were resuspended in the same buffer to a cell concentration of 1×10^7 CFU ml^{-1} by measuring the OD at 420 nm and used as inoculum.

(ii) Preparation of Pseudomonas and Paenibacillus coaggregates

The coaggregation of *Pseudomonas* and *Paenibacillus* isolates were prepared in Co-Ag buffer as described by Grimaudo and Nesbitt[14]. After coaggregation, one ml aliquot of each PGPR cells *viz*., PF-16 and Pb-16 were mixed together in 10 ml Co-Ag buffer. The mixtures were vortexed for 10 s, shaken on a rotary platform shaker for 3 min and left undisturbed at room temperature for 24 h. All Co-Ag reactions were performed in triplicate and uninoculated buffer served as control. *Helminthosporium turcicum* (AU-1) was maintained in oat meal agar (OMA) medium and used for the challenge inoculation purpose. Thick spore suspension of the same was prepared with sterile distilled water from 10 d old culture maintained in Oat Meal Agar (OMA) medium and strained through double layer muslin cloth so as to get a free suspension of conidia. The population was adjusted with the help of Haemocytometer and a spore suspension with a optimum spore concentration (50,000 spores ml^{-1}) was prepared. Then, the spore suspension was added with few drops of Tween-80 which increased the adherence capacity of the spores and acts as a sticker. The effect of different formulations of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* cells *viz*., single strain inoculation, co-inoculation and biofloc application together with challenge inoculation of *H. turcicum* on the enhancement of growth and yield in maize with special emphasis to ISR mediated biocontrol against leaf blight disease was studied. The study was conducted during September to December 2012 with maize cv. Co 1 at the polyhouse of Department of Microbiology, Faculty of Agriculture, Annamalai University, Annamalai Nagar, India.

POT CULTURE

Rectangular cement pots with 18" × 12" × 12" size were filled with 45 kg of field soil, flooded with water for 2 days and brought into fine puddle condition. The maize seeds were soaked for 30 min in the different formulations of PGPR isolates *viz*., *Pseudomonas fluorescens* (PF-16) cells alone, *Paenibacillus polymyxa* (Pb-16) cells alone, co-inoculation of *P. fluorescens* (PF-16) and *P. polymyxa* (Pb-16), and biofloc of *P. fluorescens* (PF-16) and *P. polymyxa* (Pb-16) so as to get a final population of 1×10^7 cells per seed (5 seedlings/pot). The experimental studies were performed in a randomized block design with three replications and the following were the treatments, 1) Control, 2) *Pseudomonas fluorescens* (PF-16) alone + 75% P, 3) *Paenibacillus polymyxa* (Pb-16) alone + 75% P, 4) *Pseudomonas fluorescens* (PF-16) + *Paenibacillus polymyxa* (Pb-16) co-inoculation + 75% P and 5) *Pseudomonas fluorescens* (PF-16) + *Paenibacillus polymyxa* (Pb-16) biofloc + 75% P, application. During the experimental period, the annual mean minimum and maximum temperature of the experimental area was about 25ºC and 39ºC, respectively and the mean highest and lowest humidity were 96 and 78 per cent, respectively and the mean rainfall of the area was 1500 mm. A fertilizer schedule of 100:50:50 (100% NPK ha^{-1}) was followed for the control pots, while all other treatments followed

with 75% of recommended dose of 'P' fertilizer. The entire dose of P_2O_5 and K_2O has applied basally as superphosphate and muriate of potash, respectively. Maize plants were challenge inoculated by spraying the spore suspension of *Helminthosporium turcicum* at a spore concentration of 50,000 spores ml^{-1} inoculum level on 10th DAS with an atomizer and control plant was sprayed with sterile distilled water. After spraying, the plants were covered with polythene bag for 72 h to maintain the humidity. The crop was given a hand weeding on 30th DAS and well protected against pests and diseases. The experiment was maintained under limited water supply as per the conditions prevailing in semiarid maize ecosystem. Three representative samples of plant hills in each pot were pegmarked for periodical observations. The plant height, shoot dry weight, root dry weight, chlorophyll content[15], IAA production[16], phosphorus and carbon content[17] was recorded on 45th DAS and the grain, stalk and cob yield of maize was recorded during the harvest. The reducing and non-reducing sugar was estimated according to Mahadevan and Sridhar[15] whereas the total phenol content was assayed according to Malik et al.[18] and the defense enzyme activities, such as, peroxidase (PO), poly phenol oxidase (PPO) was assayed according to Putter[19] and Matta and Diamond[20], respectively.

STATISTICAL ANALYSIS

The experimental results were statistically analyzed in randomized block design (RBD) and in Duncan's multiple range test (DMRT) as per the procedure described by Gomez and Gomez[21].

RESULTS AND DISCUSSION

The *Pseudomonas fluorescens* and *Paenibacillus polymyxa* has been emerged as the biggest, potentially the most promising group involved in biocontrol of plant diseases[7]. The application effect of the different formulations of *P. fluorescens* and *P. polymyxa* cells *viz*., single strain inoculation, co-inoculation and biofloc application on the growth and yield parameters *viz*., plant height, root and shoot dry weight, phosphorous, carbon, chlorophyll and indole acetic acid (IAA) content, grain, stalk and cob yield of maize cv. Co 1 was studied under pot culture condition (Table 1). The application of the different formulations of *Pseudomonas* and *Paenibacillus* cells was found to augment the growth and yield parameters of maize cv. Co 1 when compared to the control. Pertaining to different formulations, the application of *P. fluorescens* and *P. polymyxa* cells, as biofloc, improved growth and yield parameters of Co 1 maize to a higher level, followed by co-inoculation of *P. fluorescens* and *P. polymyxa*, *P. fluorescens* alone and *P. polymyxa* alone treatment. Interestingly, the application of "Intergeneric microbial bioflocs" comprising of *P. fluorescens* and *P. polymyxa* cells together with 75 per cent recommended P level could augment the growth and yield parameters of maize Co 1 to a higher level when compared to maize crop grown in 100 per cent recommended 'P' level without any bioinoculation and thus a saving of 25 per cent recommended 'P' fertilizers could be achieved. The biofloc application of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* recorded the maximum plant height, root length and shoot dry weight (66.36 cm, 0.357 g $plant^{-1}$, 1.638 g $plant^{-1}$, respectively), when compared to other formulations. The effect of *Paenibacillus* inoculation on the enhancement of growth and yield parameters of maize has already been reported by many researchers[22,23,24,25]. The positive effect of *Pseudomonas* and *Bacillus* coinoculation has already been reported by El-Komy et al.[26] in wheat. Neyra et al.[11] reported the positive effect of *Azospirillum* and *Rhizobium* cofloc on the enhancement of growth and yield in common bean. In the present study, the biofloc application of *P. fluorescens* and *P. polymyxa* increased the phosphorous, carbon, chlorophyll and IAA content of Co 1 maize (0.820%, 1.53%, 1.59 mg g^{-1} of leaf and 16.36 mg g^{-1} respectively), than any other formulations. The increase in dry matter production, phosphorus content, chlorophyll content, grain, stalk and cob yield of maize due to individual inoculation of either *P. fluorescens* or *P. polymyxa* has been reported by

many workers[27,28,29]. However, there were no earlier reports regarding the beneficial effect of "Intergeneric microbial coaggregates" application on plant growth stimulation not available for discussion. This is the first comprehensive report regarding the beneficial effect of *P. fluorescens* and *P. polymyxa* cells, as biofloc, on the enhancement of growth parameters in maize cv. Co 1. The studies on the application effect of different formulations of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* cells on the enhancement of ISR mediated biocontrol against *Helminthosporium turcicum* as regards to biochemical and physiological aspects revealed the highest performance of "Intergeneric microbial bioflocs" in augmenting the phenol metabolism (total phenol content and ortho-dihydroxy phenol [OD]) and defense enzyme activities (Peroxidase [PO] and polyphenol oxidase [PPO]) of maize plant whereas there was a reduction in reducing and non- reducing

Table 1

Effect of different formulations of Pseudomonas and Paenibacillus cells on the enhancement of growth and yield parameters in maize (Zea mays L.) cv. Co 1

Treatment[a]	Plant height (cm)[b]	Root dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Phosphorous content (%)	Carbon content (%)	Chlorophyll content (mg g^{-1} of leaf)	IAA content (mg g^{-1})	Grain yield (t ha^{-1})	Stalk yield (t ha^{-1})	Cob yield (g)
Control	56.42^{c}	0.257^{c}	1.489^{c}	0.687^{c}	0.62^{c}	1.07^{c}	14.82^{c}	2.222^{c}	2.53^{c}	42.86^{c}
PF-16 + 75% P*	61.25^{c}	0.305^{c}	1.522^{c}	0.745^{c}	0.83^{c}	1.28^{c}	15.54^{c}	2.425^{c}	2.962^{c}	49.27^{c}
Pb-16 + 75% P*	59.95^{d}	0.284^{d}	1.506^{d}	0.731^{d}	1.09^{d}	1.19^{d}	15.16^{d}	2.348^{d}	2.716^{d}	47.45^{d}
Co-I- PF-16, Pb-16 + 75% P**	63.70^{b}	0.332^{b}	1.54^{b}	0.76^{b}	1.37^{b}	1.41^{b}	15.97^{b}	2.67^{b}	3.142^{b}	52.72^{b}
Biofloc-PF-16, Pb-16 + 75% P***	66.36^{a}	0.357^{a}	1.63^{a}	0.820^{a}	1.53^{a}	1.59^{a}	16.36^{a}	2.742^{a}	3.206^{a}	60.29^{a}
LSD ($p \leq 0.05$)	1.681	0.017	0.026	0.021	0.16	0.08	0.27	0.09	0.127	2.91

[a] Average of three replications

[b] values followed by different letters are significantly differed at 5% level according to student "t" test

** - Individual application of Pseudomonas fluorescens (PF-16) and Paenibacillus polymyxa (Pb-16) isolates at 1×10^{7} CFU ml^{-1} inoculum level*

*** - Co-inoculation of Pseudomonas fluorescens (PF-16) and Paenibacillus polymyxa (Pb-16) isolates at 1×10^{7} CFU ml^{-1} inoculum level*

****- Biofloc of Pseudomonas fluorescens (PF-16) and Paenibacillus polymyxa (Pb-16) isolates at 1×10^{7} CFU ml^{-1} inoculum level*

Co-I – Co- inoculation

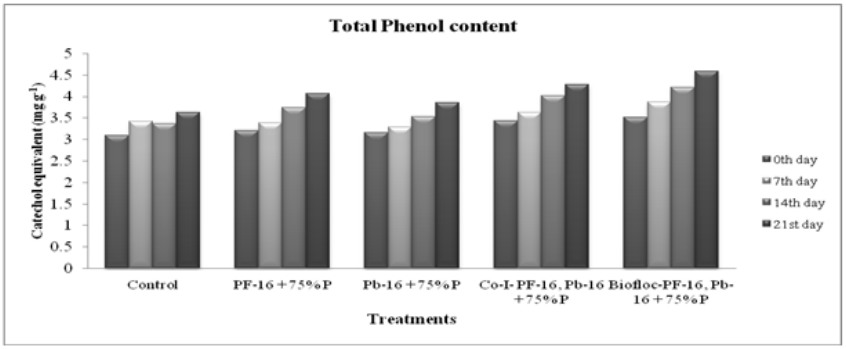

Figure 1

Changes in total phenol content of maize cv. Co 1 as influenced by different formulations of Pseudomonas and Paenibacillus cells and challenge inoculation of Helminthosporium turcicum

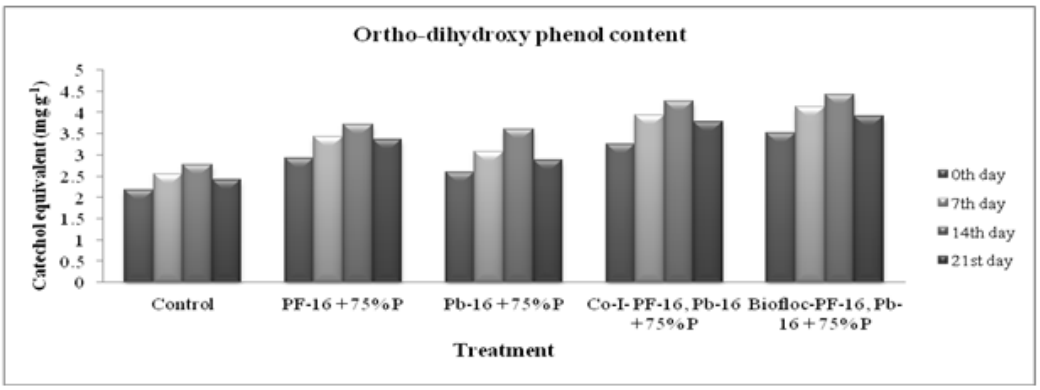

Figure 2

Changes in ortho-dihydroxy phenol content of maize cv. Co 1 as influenced by different formulations of Pseudomonas and Paenibacillus cells and challenge inoculation of Helminthosporium turcicum

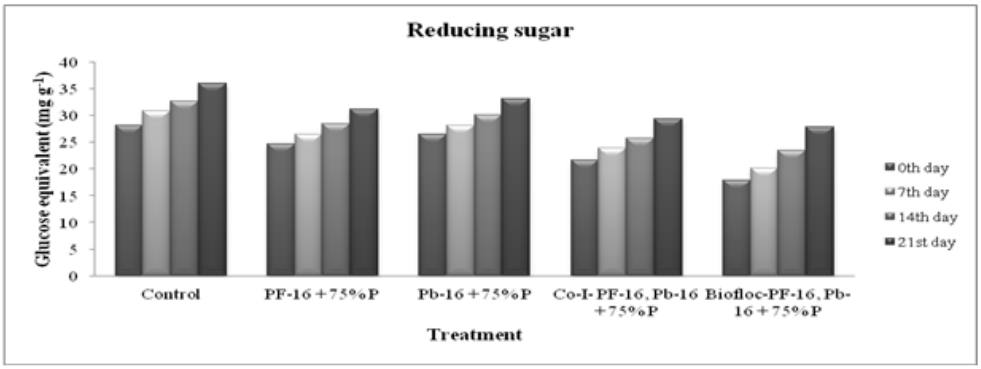

Figure 3

Changes in reducing sugar content of maize cv. Co 1 as influenced by different formulations of Pseudomonas and Paenibacillus cells and challenge inoculation of Helminthosporium turcicum

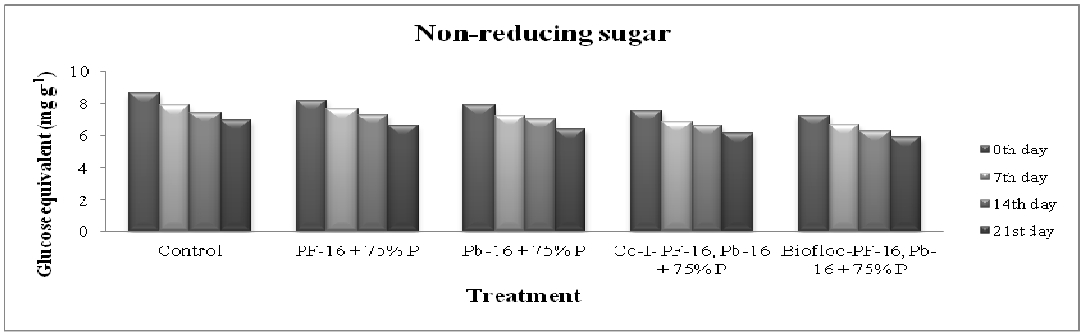

Figure 4

Changes in non-reducing sugar content of maize cv. Co 1 as influenced by different formulations of Pseudomonas and Paenibacillus cells and challenge inoculation of Helminthosporium turcicum

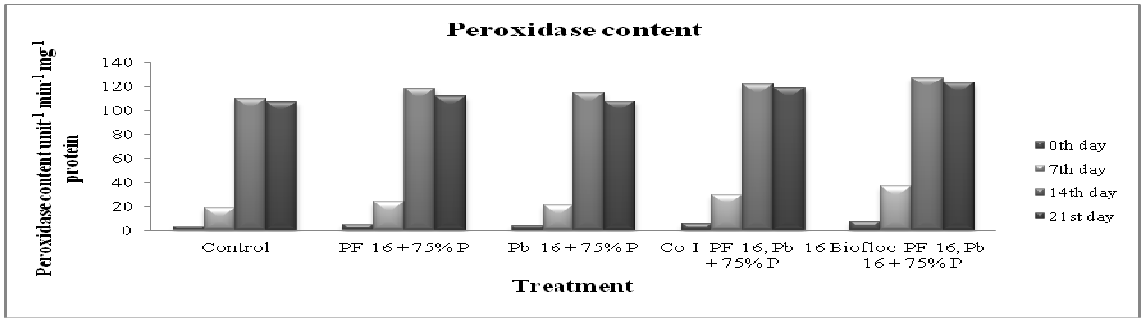

Figure 5
Changes in peroxidase content of maize cv. Co 1 as influenced by different formulations of Pseudomonas and Paenibacillus cells and challenge inoculation of Helminthosporium turcicum

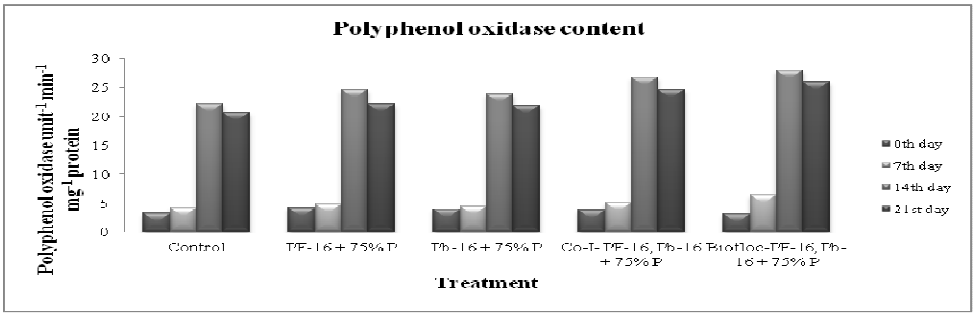

Figure 6
Changes in polyphenol oxidase content of maize cv. Co 1 as influenced by different formulations of Pseudomonas and Paenibacillus cells and challenge inoculation of Helminthosporium turcicum

sugar level which was followed by the co-inoculation of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* cells, *P. fluorescens* alone and *P. polymyxa* alone treatments (Fig 1 to Fig 6). Many experimental data pertaining to pathophysiology support the occurrence of higher phenolics level in diseased plants when compared to healthy ones. Several workers endeavored to find out a correlation between increased levels of total and ortho-dihydroxy (OD) phenol with host resistance because the post inflectional accumulation of phenols in the host plant is considered to be one of the resistant reactions[30]. It was well known that OD phenols are the most active forms of phenols and their oxidation is mediated by the enzyme polyphenol oxidase (PPO) and peroxidase (PO) and the resulting quinine production which are more toxic than phenolics and effective inhibitors of SH group of enzymes which might be inhibitor to pathogen[31]. Carbohydrates are the major source of energy and have a great influence on the incidence and development of the disease. Plant tissues containing larger amount of oxidisable carbohydrate are more prone to the invasion of pathogens than lower amount of oxidisable carbohydrates. Altered carbohydrate metabolism of the host in response to infection was studied by several workers[32,33]. Umasankari and Sekar[34] reported the increase in the phenolic content and defense enzyme activities and reduction in reducing and non-reducing sugar level in lowland rice cv. IR-50 due to *Pseudomonas fluorescens* and *Paenibacillus polymyxa* biofloc applications which eventually lead to the ISR mediated biocontrol against *Pyricularia oryzae*. In the present study, the reducing and non-reducing sugar levels were found to decrease with biofloc of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*

application together with challenge inoculation of *Helminthosporium turcicum*. The higher rate of reduction in the native level of reducing sugars may one of the vital phenomena contributing resistance to plants.

CONCLUSION

The results of the present study clearly revealed the positive role of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* cells, as biofloc application, in augmenting the ISR mediated biocontrol against *Helminthosporium turcicum* under semiarid condition. This is the first comprehensive report regarding the beneficial effect of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* cells, as biofloc, on the enhancement of induction of systemic resistance in maize cv. Co 1.

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