



Synergistic consequence of mycorrhizal fungi and zinc solubilizing bacillus sp as potential inoculant for biofortification in marigold

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Abstract

Zinc deficiency is the supreme widespread soil micronutrient scarcity all over the world. An effort to isolate, zinc solubilizing bacteria (ZSBs) and viable mycorrhizal inoculant, was tacit to comprehend the chance of solving soil zinc deficiency through microbial inoculation. The potential bacterial isolate *Bacillus sp* grown in mineral salt medium. Arbuscular mycorrhizal fungi (AMF) represent a vital group of root symbionts, given the key role they play in the improvement of plant nutrition, health, and product quality. The services provided by AMF often are facilitated by great and assorted beneficial bacterial communities, closely associated with spores, sporocarps, and extra radical mycelium, showing different functional activities, such as N₂ fixation, nutrient mobilization, and plant hormone, antibiotic, and siderophore production and also mycorrhizal establishment promotion, leading to the enhancement of host plant performance. The combined inoculation of arbuscular mycorrhizal fungi (*G. fasciculatum*) and Zinc solubilizing bacteria (*Bacillus sp*) had significantly increased the plant growth and yield of marigold. Hence, the inoculation of mycorrhizal fungi (*G. fasciculatum*) and Zinc solubilizing bacteria (*Bacillus sp*) shows immense vision in alleviating soil zinc deficiency in marigold.

Keywords: zinc solubilizing bacteria, soil zinc deficiency, mycorrhizal fungi, and bio inoculants

Introduction

Micronutrient deficiency is a foremost impediment in the steady achievement of finest crop yields. Zinc is the most commonly deprived micronutrient in worldwide crop production (Alloway 2009; Philips 2015) [1, 20], causing the utmost important nutritional sickness affecting the mainstream of the world's crop production. It affects cautiously important crops such as flower like marigold and various cereals, which are really reactive to a scarcity of zinc. Moreover, this micronutrient shortage is not readily solved by simple supplementation of fertilizers since, in alkaline pH, chemical symmetry favors the arrangement of insoluble zinc ions execution them impracticable by plants. Zinc deficiency in plants leads to shortened membrane integrity (Cakmak and Marschner 1988) [7] and union of carbohydrates, auxins, nucleotides, cytochromes, and chlorophyll and develops vulnerability to heat stress (Singh *et al.* 2005; Bharathiraja 2015; Sinha *et al.*, 2019; Bechtaoui *et al.*, 2019) [25, 2, 26]. There are numerous attitudes to transform insoluble zinc into its available forms, but these are difficult and expensive. The application of mycorrhiza is a feasible change to profitable fertilizers. It establishes synergistic effects with minute applications of chemical fertilizer, which can fairly be attributed to mycorrhiza connected microorganisms. Mycorrhiza has been exposed to anchorage valuable microorganisms in plant microorganisms has been a long-lasting goal. In earlier studies, the custom of plant growth-promoting rhizobacteria PGPR and AM fungi increased biomass and zinc contents of shoots and roots. Translocation of zinc in marigold and crossandra is also increased flower and is connected to enhanced plant-microbe interactions (Lucas *et al.* 2014; Wang *et al.* 2014; Bharathiraja and Tholkappian 2011:

Ramlakshmi and Bharathiraja 2015; Bharathiraja and Dinakar 2018; Riffat *et al* 2021; Emmett *et al* 2021) [17, 27, 2, 8, 23, 10].

Arbuscular mycorrhizal inoculant, urbanized by Department of microbiology, Faculty of Agriculture, Annamalai university, Annamalai nagar, consists of chopped dried corn roots infected with arbuscular mycorrhizal fungus, either *Glomus mosseae* or *Glomus fasciculatum* (Brown 2016; Javier and Brown 2007) [5, 14]. Vesicular-arbuscular mycorrhizal inoculant serves as a bioinoculants as well as a bio-control agent of soil-borne diseases to various crops (Nepomuceno *et al.* 2019 Riffat *et al* 2021) [18, 23]. It also assists the roots in taking up water and nutrients such as phosphorus and zinc, which are immobile. As micronutrients are required for optimal plant growth, several studies have been performed on phosphate mobilizing fungi and zinc solubilizing bacteria. About 96 to 99 per cent of the applied zinc is rehabilitated to different insoluble forms depending upon the soil types and physico-chemical reactions of the soil. The solubility of zinc is extremely dependent on soil pH and moisture.

Zinc occurs in soil as sphalerite, olivine, hornblende, augite and biotite. However, availability of zinc from these sources is guided by numerous factors among which biochemical actions of rhizosphere microorganisms play a vital role in converting such unavailable sources into available form (Bhupinder *et al.*, 2005). Microorganisms play a key role in zinc solubilization. Some species of rhizobacteria are capable of mobilizing zinc in available form in soils. Mineral zinc solubilization by microbes which enhances crop growth and yield. Zinc solubilising bacteria are competent of solubilizing ZnO, ZnCO₃ and zinc phosphate through production and secretion of organic acids.

Materials and Methods

Isolation and characterization of AM fungi and zinc solubilizing bacteria from rhizosphere soil of marigold

The present investigation was carried out in the Department of microbiology, Faculty of Agriculture, Annamalai University, Annamalai nagar, The rhizosphere soils of marigold sample were collected from fifteen different locations in Cuddalore District of Tamilnadu. Five different AM fungal species viz., *Glomus fasciculatum*, *Glomus mosseae*, *Gigaspora margarita*, *Acaulospora laevis* and *Glomus versiforme* were isolated, characterized and identified under stereo zoom microscope according to Gerdemann and Trappe (1974) [11]. Isolated AM fungi are screened for the competence by root colonization percentage, AM fungal spore numbers, acid phosphatase and alkaline phosphatase activity in soil. All the five AM fungal species colonized the roots of marigold. However, the degree of root infection and colonization varied significantly between them. The response of marigold in terms of root colonization by AM fungi was the maximum with *Glomus fasciculatum* followed by *Glomus mosseae*, *Gigaspora margarita*, *Glomus versiforme* and *Acaulospora laevis* in soils. Acid phosphatase and alkaline phosphatase activities were also the highest in *Glomus fasciculatum*

Zinc solubilizing bacteria were isolated from collected soil samples by serial dilution plate count method using mineral salt medium (Di Simine *et al.*, 1998; Dinakar and Bharathiraja 2018; Emmett *et al.*, 2021) [9, 8, 10] which is a selective medium for isolation of zinc solubilizers. Agar medium containing 0.1 % mysterious zinc compound (ZnO). The plates were incubated at room temperature (30±1°C) for 3 days and the colonies exhibiting clear zones were selected, purified by four-way streak plate method. The width of zone of solubilization was deliberate and expressed in centimeter and the selected isolates were conserved on agar slants for further use.

Identification and characterization of the bacterial isolates

All the selected isolates were examined for the colony morphology, cell shape, and gram Reaction as per the standard procedures given by Anonymous (1957) and Barthalomew and Mittewer (1950). Fifteen zinc solubilizing bacteria isolates were screened for zinc solubilizing efficiency, Starch hydrolysis, Casein hydrolysis, n, Catalase test, Hydrogen sulphide production, Urease test, producing potential of Indole acetic acid (IAA) and Gibberellic acid (GA₃) and Siderophore. Among fifteen isolates, *Bacillus sp* was found to be the most efficient isolate in solubilizing various insoluble ZnO, ZnCO₃ and zinc phosphate through production and secretion of organic acids. Based on the above screening tests, the isolates *Glomus fasciculatum* and *Bacillus sp* were found to be most efficient strains and selected for further studies

Pot culture experiment

A pot culture trial was conducted to assess the synergistic effect of AM fungi and Zinc solubilizing bacillus on the growth enhancement and flower yield of marigold. The annual mean least and highest temperature of experimental area is 25°C and 39°C respectively and the mean maximum and lowest relative humidity was 96 and 78 percent respectively the mean annual rainfall of this area is 1500mm. The cement pots of 1' X 2' X 2' were filled with well drained loamy soil

@ 12kg pot⁻¹ (soil pH 7.0 to 8.5, available N 210 kg ha⁻¹ and available phosphorus 16.71 kg ha⁻¹). The recommended dose of inorganic fertilizer 90:90:750 kg/ha respectively. AM fungal inoculum was achieved by 10g inoculum near the roots. Thirteen treatments were tried with inoculation of AM fungi and Zinc solubilizing bacteria three replications were maintained for each treatment and uninoculated control without inoculum was maintained under complete randomized block design.

The treatments schedule was as follows.

Crop: Marigold
Varieties: Pusa basanthi
Design: CRD
Replication: Three

Treatment details

T₁: Control
T₂: RDF
T₃: *G. fasciculatum*
T₄: Zinc solubilizing bacteria
T₅: 100% NPK + *G. fasciculatum*
T₆: 100% NPK + Zinc solubilizing bacteria
T₇: 100% NPK + *G. fasciculatum* + Zinc solubilizing bacteria
T₈: 75% NPK + *G. fasciculatum*
T₉: 75% NPK + Zinc solubilizing bacteria
T₁₀: 75% NPK + *G. fasciculatum* + Zinc solubilizing bacteria
T₁₁: 50% NPK + *G. fasciculatum*
T₁₂: 50% NPK + Zinc solubilizing bacteria
T₁₃: 50% NPK + *G. fasciculatum* + Zinc solubilizing bacteria

Beneficial interaction of AM fungi and Zinc solubilizing bacteria on the biometric observation of marigold:

Plant height

The plant height was recorded at 30, 60 and 90 DAT. The height was precise from the ground level to the tip of the growing point. The mean values were calculated and recorded in cm.

Dry matter production

The plant samples were collected from each treatment and their dry weight was determined by drying the samples in hot air oven at 60°C till a constant weight was obtained. The mean values were calculated and recorded in g plant⁻¹

Estimation of xanthophyll content

Xanthophyll content were extracted by grinding the leaf tissue with a mortar and pestle using ammoniacal acetone. The resulting extracts were centrifuged at 3000 rpm for 3 min. The total xanthophyll contents were determined by ultraviolet-Vis spectrophotometry (Beckman DU-50 spectrophotometer; Beckman Instruments, Inc., Fullerton, CA). The absorbance of the solution was measured at 470, 647, and 664 nm. Formulae and extinction coefficients used for the determination of leaf pigments were described by Lichtenhaler and Wellburn (1983) [16]. The content of the xanthophyll was expressed in g Kg⁻¹

Statistical analysis

The data recorded on various characters were statistically analysed as per the method by Gomez and Gomez (1984) [13].

Results and Discussion

Screening of AM fungal isolates for colonization spore population and phosphatase activity in marigold

The isolated spores were characterized according to Gerdmen and Trappe (1974) [11] and the five AM fungal species viz., *Glomus fasciculatum*, *Glomus mossae*, *Gigaspora margarita*, *Glomus versiforme* and *Acaulosporala evis* were originate to be there in all the soil types. Pot culture experiment was conducted to screen the five AM fungal isolates viz., *G. fasciculatum*, *G. mossae*, *Gi. margarita*, *A. laevis* and *G. versiforme* for the root colonization percentage, spore number (per 100g of rhizosphere soil), acid and alkaline phosphatase activity in marigold plant exhibited in Table 1. The per cent root colonization, spore number (100g⁻¹ of rhizosphere soil), acid and alkaline phosphatase enzyme activities were found to be higher in *G. fasciculatum* inoculated marigold plants compared to other isolates *G. mossae*, *A. laevis*, *G. margarita* and *G. versiforme* inoculated plants. The highest root colonization

(77.23%) and spore number (182.43/100g of rhizosphere soil) were recorded in *G. fasciculatum* inoculated plants followed by *G. mossae* (63.20% and 177.23/100g rhizosphere soil), *A. laevis* (57.16% and 171.83/100g rhizosphere soil) *Gi. margarita* (53.16% and 167.68/100g rhizosphere soil) and *G. versiforme* (48.20% and 165.45/100g rhizosphere soil) on 90 DAT. Similar results were observed in several authors Giovannin *et al.*, 2020 [12]; Karray *et al.*, 2020 [15]; Sharma *et al.*, 2020 [24]; Pecundo *et al.*, 2021 [19].

The highest acid and alkaline phosphatase activities were recorded in *G. fasciculatum* inoculated roots as 29.73 and 28.36 µg 24 h⁻¹ 10g of root) followed by *G. mossae* (29.53 and 27.55 µg 24 h⁻¹ 10g of root). The other isolates were able to exhibit between 26.43 - 29.53 and 23.20 - 28.36 µg 24 h⁻¹ 10g of root respectively for acid and alkaline phosphatase activity. In general, the acid phosphatase activity was more when compared to alkaline phosphatase activity.

Table 1: Screening Table of AM fungal isolates for colonization spore population and phosphatase activity in marigold

S. No.	AM fungal isolate	Root colonization (%)			AM fungal spore number (100 g ⁻¹ of rhizosphere soil)			Acid phosphatase activity (µg/ 24 hrs ⁻¹ 10g ⁻¹ of root)			Alkaline phosphatase activity (µg/ 24 hrs ⁻¹ 10g ⁻¹ of root)		
		30 DAT	60 DAT	90 DAT	30 DAT	60 DAT	90 DAT	30 DAT	60 DAT	90 DAT	30 DAT	60 DAT	90 DAT
1	<i>Glomus mosseae</i>	41.23	56.43	63.20	144.20	166.90	177.23	26.56	27.60	29.53	25.30	26.59	27.55
2	<i>Glomus fasciculatum</i>	53.20	63.26	77.23	153.17	170.42	182.43	27.51	28.73	29.73	26.70	28.03	28.36
3	<i>Glomus versiforme</i>	26.27	38.22	48.20	127.73	158.03	165.45	23.43	25.20	26.43	22.17	22.86	23.20
4	<i>Acaulospora laevis</i>	37.50	50.30	57.16	139.46	163.86	171.83	25.30	26.40	28.23	24.06	25.10	25.46
5	<i>Gigaspora margarita</i>	30.23	43.36	53.16	132.43	158.93	167.68	24.46	26.01	27.21	22.56	24.63	23.65
	SE	1.63	1.09	1.03	1.15	0.53	1.15	0.30	0.16	0.34	0.43	0.45	0.63
	CD (p = 0.05)	3.13	2.10	2.02	2.25	1.51	2.46	0.73	0.42	0.79	0.93	0.93	1.35

Isolation of zinc solubilizing bacteria (ZSB) from rhizosphere soils of marigold

Isolation and characterization of Zinc solubilizing bacteria from rhizosphere soil of marigold. Serially dilute the soil sample from rhizosphere soil of marigold, 1 ml of aliquot from 10⁻⁵ and 10⁻⁶ was transferred in to specific media zinc oxide on agar media supplemented with zinc oxide at 0.1 per cent After incubation of 5 days to observe hallow zone around the colonies. There are 15 isolates were isolated and designated as ZSB1 to ZSB15 (Table.2) the morphological and biochemical characterized by size, shape, gram's reaction, catalase activity, H₂S production and methyl red. Based on the different biochemical characters to the genus level identify 2 genera like *Pseudomonas* sp. and *Bacillus* sp. The *in vitro* production of phytohormones such as indole acetic acid (IAA) and gibberellic acid (GA₃) by Zinc solubilizing bacteria were estimated. The gibberellic acid production by Zinc solubilizing bacteria was determined by following the method of Borrow *et al.* (1955) [6]. Siderophore production by the Zinc solubilizing bacteria was estimated by the method described by Reeves *et al.* (1983). All the fifteen isolates examined for their ability to solubilise zinc oxide on agar media supplemented with zinc oxide at 0.1 per cent. The diameter of zone of solubilization formed by the isolates ranged from 0.27 to 1.70 cm at 72 hours after incubation (HAI). Among the isolates ZSB-1 recorded maximum solubilization zone (1.60 cm diameter) followed by ZSB-2 (1.60 cm) and ZSB-12 (1.40 cm). However, the isolate ZSB-15 showed the least solubilization zone of 0.27 cm diameter.

Table 2: Zone of solubilisation by Zinc solubilizing bacterial isolates

Sl. No	Isolates	Zone of solubilisation (diameter)
	ZSB1	1.70
2	ZSB2	1.90
3	ZSB3	1.20
4	ZSB4	0.70
5	ZSB5	0.70
6	ZSB6	1.20
7	ZSB7	0.80
8	ZSB8	0.70
9	ZSB9	1.20
10	ZSB10	1.20
11	ZSB11	1.30
12	ZSB12	0.30
13	ZSB13	0.50
14	ZSB14	0.40
15	ZSB15	0.28

Beneficial interface of AM fungi and Zinc solubilizing bacteria on the biometric surveillance of marigold

The treatment (T₁₀: 75% NPK + *G. fasciculatum*+ Zinc solubilizing bacteria) recorded maximum plant height of 21.25 cm, 40.08 cm and 60.28 cm on 30, 60 and 90 DAT respectively. It was followed by T₇ (100% NPK + *G. fasciculatum*+ +Zinc solubilizing bacteria) as 20.33 cm, 38.90 cm and 57.13 cm (Table 3). The treatments T₃ (*G. fasciculatum* alone) and T₄ (Zinc solubilizing bacteria) were observed the plant height of 47.39 cm and 46.73 cm on 90 DAT. whereas, the treatment T₂ (100% NPK) recorded 51.00 cm on 90 DAT and found to be better than individual application of either *G. fasciculatum* or zinc solubilizing bacteria. These results are accordance with those of Riffat *et al.*, 2021 [23]; Bharathiraja and Tholkappian 2011:

Ramlakshmi and Bharathiraja 2015 [2]. It was observed that plant height was significantly improved in all the treatments (T₅ –T₁₃) receiving *G. fasciculatum* and Zinc solubilizing bacteria either individually or combination along with graded levels (100, 75 and 50%) of inorganic fertilizer. The *Bacillus sp* have been well documented for their potential to solubilize insoluble zinc source and influence the growth and yield for example Ramesh *et al.*, (2014). The dry matter production of marigold was significantly improved by the inoculation of *G. fasciculatum* and zinc solubilizing bacteria along with graded levels of inorganic P and 100% N & K fertilizers as compared to control. The values regarding on dry matter production on 30, 60 and 90 DAT. The xanthophyll content was measured at the time of harvest stage of marigold. The significant effect of *G. fasciculatum*+ Zinc solubilizing bacteria recommended dose of NPK fertilizers over control was observed. The maximum xanthophyll content was found in (T₁₀) 75% NPK + *G. fasciculatum*+ Zinc solubilizing bacteria (40.14 gkg⁻¹ and 19.66 kgha⁻¹) which was followed by T₇ (38.01 gkg⁻¹ and 17.80 kgha⁻¹). The minimum amount of xanthophyll content was recorded in control

(30.42 gkg⁻¹ and 9.73 kgha⁻¹). Similar result were observed in several authors Giovannin *et al.*, 2020 [12]; Karray *et al.*, 2020 [15]; Sharma *et al.*, 2020 [24]; Pecundo *et al.*, 2021 [19]

The growth character *viz.*, plant height, number of laterals and number leaves have been influenced by NPK content. Due to the fact, that nitrogen is the major constituents of amino acid and protein which occupy a major portion of the protoplasm being important for the crop growth and development of marigold. The results of the present investigation were in accordance with the studies on Khan *et al.* (2004); Bharathiraja and Tholkappian 2011: Ramlakshmi and Bharathiraja 2015 [2]; Riffat *et al* 2021 [23].

In this experiment, potential Zinc Solubilizing *Bacillus* and *Glomus fasciculatum* were isolated from the rhizosphere soils of marigold. The isolates exhibited halozones in mineral salt agar medium with a solubilization index ranged from 0.27 to 1.70 cm at 72 hours after incubation (HAI). Among the isolates ZSB-1 recorded maximum solubilization zone (1.60 cm diameter) followed by ZSB-2 (1.60 cm) and ZSB-12 (1.40 cm). However, the isolate ZSB-15 showed the least solubilization zone of 0.27 cm diameter.

Table 3: Effect of *G. fasciculatum* and Zinc solubilizing bacteria on the plant height and dry matter production of marigold

Sl. No	Treatment	Plant height (cm)			Dry matter production (g plant ⁻¹)		
		30DAT	60DAT	90DAT	30DAT	60DAT	90DAT
T1	Control	15.00	26.40	46.60	5.78	12.80	26.28
T2	RDF	19.40	32.08	51.00	5.93	18.20	32.26
T3	<i>G. fasciculatum</i>	15.26	26.50	47.39	5.89	14.60	30.60
T4	Zinc solubilizing bacteria	15.24	26.45	46.73	5.87	14.40	30.40
T5	100% NPK + <i>G. fasciculatum</i>	17.20	36.00	54.10	6.93	22.88	35.50
T6	100% NPK + Zinc solubilizing bacteria	17.00	35.10	53.10	6.80	22.40	34.28
T7	100% NPK + <i>G. fasciculatum</i> + +Zinc solubilizing bacteria	20.33	38.90	57.13	8.63	24.13	45.80
T8	75% NPK + <i>G. fasciculatum</i>	18.03	36.90	56.00	7.83	22.96	38.30
T9	75% NPK + Zinc solubilizing bacteria	17.99	35.53	55.18	7.37	20.50	36.46
T10	75% NPK + <i>G. fasciculatum</i> + Zinc solubilizing bacteria	21.25	40.08	60.28	9.87	25.46	50.20
T11	50% NPK + <i>G. fasciculatum</i>	16.80	34.20	53.00	5.97	19.20	34.26
T12	50% NPK + Zinc solubilizing bacteria	16.03	33.90	51.33	5.65	19.10	32.06
T13	50% NPK + <i>G. fasciculatum</i> + Zinc solubilizing bacteria	18.33	37.36	56.63	7.89	23.26	39.66
	SE	0.44	0.56	1.53	0.45	0.65	1.96
	CD (p = 0.05)	0.92	1.16	3.13	0.93	1.32	3.96

Table 4: Effect of *G. fasciculatum* and Zinc solubilizing bacteria on the xanthophyll content of marigold

Sl. No	Treatment	Xanthophyll content	
		gkg ⁻¹	kgha ⁻¹
T ₁	Control	30.42	9.72
T ₂	RDF	31.00	11.60
T ₃	<i>G. fasciculatum</i>	32.10	12.50
T ₄	Zinc solubilizing bacteria	32.00	12.42
T ₅	100% NPK + <i>G. fasciculatum</i>	34.94	14.80
T ₆	100% NPK + Zinc solubilizing bacteria	34.60	14.60
T ₇	100% NPK + <i>G. fasciculatum</i> + +Zinc solubilizing bacteria	38.01	17.80
T ₈	75% NPK + <i>G. fasciculatum</i>	36.94	16.56
T ₉	75% NPK + Zinc solubilizing bacteria	35.04	15.40
T ₁₀	75% NPK + <i>G. fasciculatum</i> + Zinc solubilizing bacteria	40.14	19.66
T ₁₁	50% NPK + <i>G. fasciculatum</i>	33.98	12.80
T ₁₂	50% NPK + Zinc solubilizing bacteria	33.46	12.60
T ₁₃	50% NPK + <i>G. fasciculatum</i> + Zinc solubilizing bacteria	37.03	16.60
	SE	1.03	0.86
	CD (p = 0.05)	2.10	1.80

Conclusion

The potential isolates released a significant amount of zinc in mineral salt medium amended with zinc oxide as the insoluble zinc source. Isolated AM fungi are screened for

the competence by root colonization percentage, AM fungal spore numbers, acid phosphatase and alkaline phosphatase activity in soil. All the five AM fungal species colonized the roots of marigold. However, the degree of root infection

and colonization varied significantly between them. The response of marigold in terms of root colonization by AM fungi was the maximum with *Glomus fasciculatum* followed by *Glomus mosseae*, *Gigaspora margarita*, *Glomus versiforme* and *Acaulospora laevis* in soils. Acid phosphatase and alkaline phosphatase activities were also the highest in *Glomus fasciculatum*. The isolates also significantly increased plant height of marigold in soils with insoluble zinc, with *Bacillus sp* and *Glomus fasciculatum* showing similar plant height, dry matter production and xanthophylls content as the positive control. Thus, the use of potential Zinc Solubilizing Bacillus and *Glomus fasciculatum* has great potential in addressing zinc deficiency as well growth and yield attributes of marigold.

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