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Original Research Article

Efficiency study of phosphate solubilizing bacterial and fungal species and effects on moth gram seed germination

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ABSTRACT

The present work aimed to check the efficiency of various phosphate solubilizing microorganisms and their effect on seed germination. In this study, nine different strains of fungi and bacteria were used. *Aspergillus awamori NCIM 861, Aspergillus niger NCIM 1248, Aspergillus oryzae NCIM 641, Bacillus megaterium NCIM 2087, Bacilus polymyxa NCIM 2846, Bacillus sphericus NCIM 2478, Pseudomonas striata NCIM 2847, Trichoderma viridae NCIM 1051, Trichoderma ressie NCIM 992.* Among these five strains were selected for seed germination studies based on TCP (Tri Calcium Phosphate) and RP (Rock Phosphate) solubilization. Phosphate solubilization was carried out with Pikovskaya medium for the determination of the efficiency of all the PSMs. According to the results obtained from TCP and RP solubilization and seed germination experiments, it is found that *Ps. striata* is more efficient among all the used species in the study of phosphate solubilizers.

1. Introduction

Phosphorus (P) is an essential nutrient for plants because of its vital role in photosynthesis and many energy transformation processes. Many microorganisms are involved in a range of processes that solubilize the fixed form of phosphorus in the soil viz. *Bacillus, Pseudomonas, Aspergillus, Trichoderma, Penicillium.*[1] Phosphate-solubilizing bacteria, belonging to the group of plant growth-promoting rhizobacteria (PGPR), mobilize deposits of insoluble phosphates in the soil.[2]

PSM supports plant growth through the production of siderophore, increases the efficiency of nitrogen fixation, acts as a biocontrol against plant pathogens via the production of antibiotics, hydrogen cyanate (HCN), and antifungal metabolites.[3]

In particular, soil microorganisms are effective in releasing P from inorganic phosphate through solubilization[4–6] and from organic pools of total soil P by mineralization.[7,8] In 2013, Gaind reported that *Pseudomonas striata* showed high potential when evaluated for phytate mineralization and solubilization of tricalcium, rock, ferric, and aluminum phosphate. Chromatographic analysis of cell-free culture filtrate showed the presence of tartaric acid, malic acid, citric acid, succinic, and gluconic acid. Tartaric acid was effective in the solubilization of TCP.

However, approximately 95–99% insoluble phosphate is present in the soil and hence cannot be utilized by the plants.[9] A large proportion of fertilizer phosphorus is quickly transferred to the insoluble form.[10] Therefore, a very little percentage of the applied phosphate is used, making continuous application necessary.[7] The application of PSMs in the field has been reported to increase crop yield.[11] The mechanisms such as lowering of pH due to acid production, ion chelation and exchange reactions in the growth environment[12] and protons, hydroxyl ions and CO_2[13] carry out the phosphate solubilization by PSMs. Fungi have a greater ability to solubilize insoluble

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phosphate than bacteria.[14]

In the present study, the efficiency of different PSMs was confirmed by observing phosphate solubilization in Pikovskaya broth and agar medium. The germination studies on moth gram seeds were carried out with efficient PSMs.

2. Materials and Methods

All the procedures and experiments included in the present research work are carried out at Rashtriya Chemicals and Fertilizers Limited, Trombay, Mumbai.

2.1. Microbial cultures

The fungal and bacterial cultures used were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratories (NCL) India.

2.2. Media

Pikovskaya media was used for the revival of PSMs and to check phosphate solubilization.

2.3. Phosphate solubilization on Pikovskaya's medium

Spot inoculation on Pikovskaya's media was done and the plates were incubated at 27^0C to 30^0C. The petriplates were observed for the zone of solubilization after overnight incubation. The zones were measured by using a scale.

2.4. Phosphate solubilization by microorganisms in liquid media

Pikovskaya's broth containing 1% RP and 0.5% TCP were inoculated separately with bacterial and fungal spores and incubated on a rotary shaker for 7 days at room temperature. After 7 days filtration was carried out with Whatmann filter paper No. 42. Filtrate about12-15 ml were collected in clean 50 ml flasks separately. The broth inoculated with bacteria was centrifuged in a refrigerated centrifuge at 15000 rpm for 30 min. Supernatant from each broth collected separately in chemically clean 50 ml flasks. Finally, 12-15 ml supernatant was obtained. The pH of all the supernatant filtrates and blank was measured on pH meter (JENCO-6173).

2.5. Quantitative estimation of solubilized phosphorus (P_2O_5) by blue colour method.[15]

Each sample of supernatant and filtrate was taken about 5 ml in chemically clean, labeled 20 ml test tubes separately. Then 5 ml of Chloromolybdic acid reagent was added per tube. To this 1 ml of stannous chloride, reagent added suddenly. The intensity of the developed blue colour was measured on a spectrophotometer at 600 nm after proper mixing. The O.D. was extrapolated on the standard graph of 2 ppm phosphorus and the concentration of solubilized phosphate is determined.

2.6. Cultivation of efficient PSMs in liquid media for germination studies

Five species of efficient PSMs viz. *Aspergillus niger, Aspergillus awamori, Trichoderma viridae, Bacillus polymyxa,* and *Pseudomonas straita* were inoculated separately in 500 ml flasks containing 200 ml of P-media broth and incubated on a rotary shaker at room temperature for 2 days. After 2 days these cultures were diluted with sterilized deionized water to 1% and 2% concentration.

2.7. Seed germination studies

The moth gram (*Vigna aconitifolia*) seeds were first treated with 1% Mercuric Chloride ($HgCl_2$) solution for 1-2 minutes for surface sterilization. Then the seeds were rinsed three times with sterile demineralized water. 1% and 2% concentration of *Aspergillus niger, Aspergillus awamori, Trichoderma viridae, Bacillus polymyxa,* and *Pseudomonas straita* were taken in sterilized petriplates separately. In these petriplates the 5 seeds were soaked for 30 mins. In another plate, five seeds were soaked in sterilized deionized water and used as a control in the experiment of seed germination. After this seeds were placed in sterilized petridishes containing sterilized filter paper drenched in sterilized deionized water and these plates were kept for germination. Observations were noted at different times of interval till the end of the third day.

3. Results

The qualitative results of phosphate solubilization in the form of the zone of solubilization are shown in Table 1. The largest zone observed in the case of *A. awamori* (42 mm) followed by *A.oryzae* (36mm), *T.ressie* (32 mm), and *T.viridae* (26 mm). The Bacterial cultures showed moderate to very small zones of solubilization. The most prominent exhibited by *Ps.straita* (11 mm), followed by *B.polymyxa* (10 mm), *B.sphericus* (8 mm), *B.megaterium* (7 mm), and intermediate zone of solubilization shown by *A. niger* (15 mm).

Table 1: Results of phosphate solubilization

S. No.	Strain name	Diameter of the zone (mm)
1	*Asp.awamori*	42
2	*Asp.oryzae*	36
3	*Asp.niger*	15
4	*B.megaterium*	7
5	*B.Polymyxa*	10
6	*B.sphericus*	8
7	*Ps.straita*	11
8	*T.ressie*	32
9	*T.viridae*	26

The results of the quantitative estimation are shown in Table 2. Figure 2 are showing the graphs for estimation P_2O_5 in Rock phosphate and Tri Calcium Phosphate, and seed germination respectively. Moth gram seed germination with *Ps. Straita* can be seen in Figure 3. The concentration of P_2O_5 varies in the case of RP and TCP with each microorganism. The TCP solubilized *by Pseudomonas straita (*620 mg/L), *A.niger* (190 mg/L), *A.oryzae* (91 mg/L), *A.awamori* (86mg/L), *T.viridae* (94 mg/L), *T.ressie* (27 mg/L), *B.megaterium* (63 mg/L), *B.Sphericus* (34 mg/L). *B.polymyxa* (8.5 mg/L). In the case of RP the solubilization was found to be - *B.polymyxa* (86 mg/L), *T.viridae* (85 mg/L), *Ps.Straita* (63 mg/L), *A.awmori* (50 mg/L), *B.megaterium* (4.5 mg/L), *B.sphericus* (8.8mg/L), *T.ressie* (10.5mg/L), *A.oryzae* (10 mg/L), *A.niger* (19 mg/L).

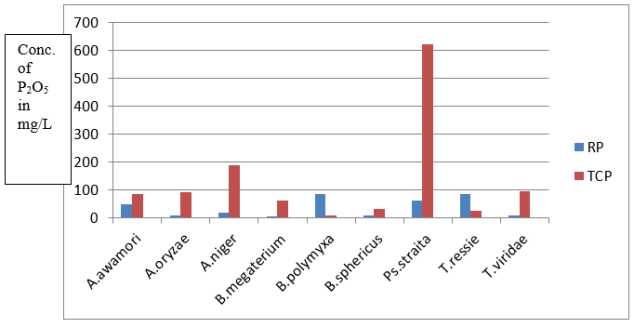

Fig. 1: Graph of quantitative estimation of P_2O_5

Moth gram seeds germination showed the following results with different PSMs (Table 3)

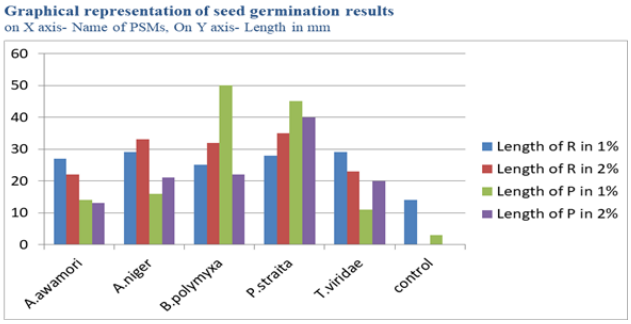

Fig. 2: Graph of seed germination (R- Radicae, P- Plumule)

P.straita - length of radical and plumule recorded 28 mm and 45 mm respectively in 1% concentration, and 32 mm and 40 mm in 2% respectively. In *B.polymyxa* radical - 32 mm and plumule - 22 mm 2% concentration and in 1% 50 mm – radical and 25 mm - plumule. The length of radical and plumule in control is noted 14 mm and 03 mm respectively and for *A.niger, A.awamori, T. viridae, T.viridae,* (Plumule - 11 mm in 1%) and *A.awamori* (radical - 22 mm at 2%) on treatment.

4. Discussion

Phosphate-solubilizing bacteria play a vital role in plant nutrition through the increase in P uptake by the plant. Their use as PGPR is an important contribution to the biofertilization of crops.[16] Application of advantageous microbes to seed for use in agriculture, forestry, and horticulture to perform specific functions, such as nitrogen fixation, phosphate solubilization, plant-growth promotion, biological control of plant pathogens and pests can benefit the overall crop growth.[17]

The results of Phosphate solubilization of RP and TCP are shown in Table 2. According to this; the concentration of P_2O_5 varies in the case of RP and TCP with each microorganism.

Halo zones production on solid media and efficient release of phosphate is due to the release of several organic acids like citric, keto, glyoxalic succinic butyric, and malic.[18] The halo zone formation around the bacterial colonies might be owed to the production of organic acids or because of the production of polysaccharides or due to the activity of phosphatase enzymes of phosphate solubilizing bacterial strains.[19–22]

Many isolates which, did not show any zone of solubilization on agar plates have released phosphate from RP when inoculated in the liquid medium.[23,24]

In the present study TCP *Pseudomonas straita* solubilized a very high amount (620 mg/L), it is much greater (12.4%) than any other strain of PSM used in our study. Some of them also solubilized TCP efficiently viz. *A.niger* (190 mg/L), *A.oryzae* (91 mg/L), *A.awamori* (86mg/L), *T.viridae* (94 mg/L), followed by *T.ressie* (27 mg/L), *B.megaterium* (63 mg/L), *B.Sphericus* (34 mg/L). *B.polymyxa* solubilized TCP in very low amount (8.5 mg/L). In case of RP *B.polymyxa* solubilized much more amount of phosphate (86 mg/L) followed by *T.viridae* (85 mg/L), *Ps.Straita* (63 mg/L), *A.awmori* (50 mg/L), but very low concentration of solubilized Phosphate is shown by *B.megaterium* (4.5 mg/L). Other strains also showed quite low range of solubilization of RP eg. *B.sphericus* (8.8mg/L),*T.ressie* (10.5mg/L), *A.oryzae* (10 mg/L), *A.niger* (19 mg/L).

Tricalcium phosphate solubilization by *Pseudomonas putida* was estimated along with a temperature range (4-28^{0}C), and maximum activity (247 mg/ml) was recorded at 21^0 C after 15 days of incubation. The phosphate solubilizing activity concurred with a concomitant drop off in the pH of the medium. The isolate also exhibited antifungal activity against phytopathogenic fungi in petri dish assays and produced chitinase, ss-l, 3-glucanase, salicylic acid, siderophore, and hydrogen cyanide.[25] *Pseudomonas fluorescens* K - 34 solubilized TCP and

Table 2: Results of quantitative estimation

S.No.	Strain Name	Accession No.	Solubilized Rock Phospate (Jordan)		Solubilized Tricalcium Phosphate	
			pH	Conc.(mg/L)	pH	Conc.(mg/L)
1	*A.awamori*	NCIM-861	5.0	50	5.4	86
2	*A.oryzae*	NCIM-641	5.8	10	5.8	91
3	*A.niger*	NCIM-1248	5.6	19	4.9	190
4	*B.megaterium*	NCIM-2087	5.8	4.3	5.6	63
5	*B. polymyxa*	NCIM-2846	5.6	86	6.2	8.5
6	*B.sphericus*	NCIM-2478	6.0	8.8	6.2	34
7	*P.straita*	NCIM-2847	5.5	63	5.4	620
8	*T.ressie*	NCIM-992	5.9	85	5.6	27
9	*T.viridae*	NCIM-1051	5.7	10.5	5.7	94

Table 3: Seed germination

S.No.	Culture	Days After Inoculation						Conc. (%)
		1		2		3		
		R	P	R	P	R	P	
1	*A.awamori*	18	00	22	03	17	14	1
		11	00	19	04	22	13	2
2	*A.niger*	20	05	25	08	29	16	1
		23	00	28	16	33	21	2
3	*B.polymyxa*	15	00	20	05	25	50	1
		13	00	18	02	32	22	2
4	*Ps.straita*	15	00	24	01	28	45	1
		18	00	30	04	35	40	2
5	*T.viridae*	19	05	25	06	29	11	1
		20	06	10	16	23	20	2
6	*Control*	08	00	13	00	14	03	-

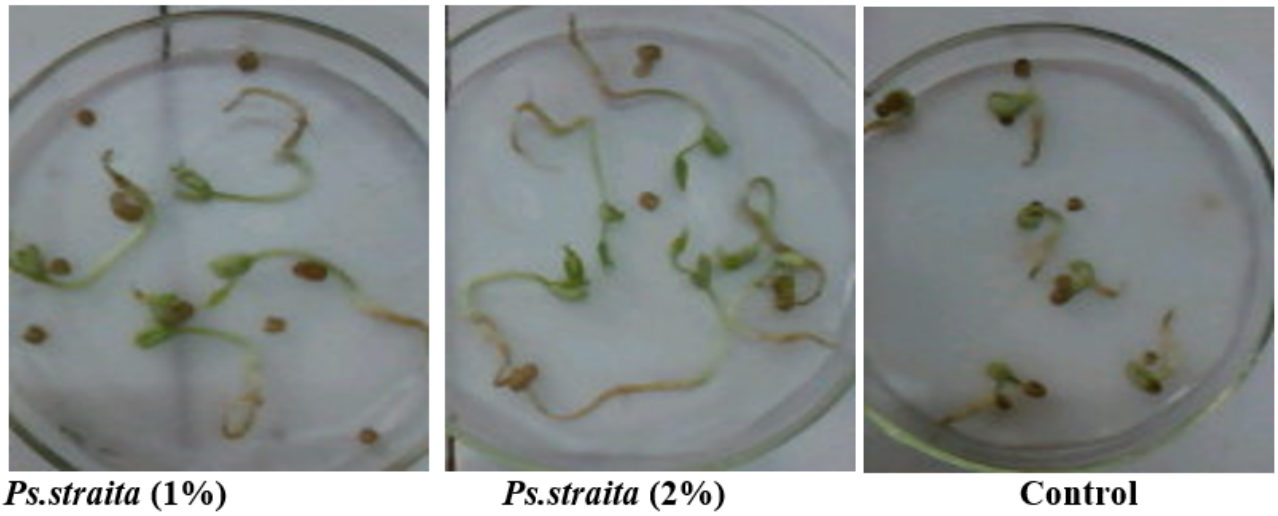

Fig. 3: Moth gram seed germination with *Ps.straita* and in control after 3 days

produced a substantial amount of soluble phosphorus (968.5 mg / l) in Pikovskaya broth as compared to others and exhibited the production of indole acetic acid (IAA), siderophore, cell wall degrading enzyme activities and growth inhibition against fungal and bacterial pathogens.[26] The root development and plant biomass were highly correlated with the higher availability of P. The PSB application may also have some other beneficial effects, such as phytohormone production.[27]

The biosynthesis of growth-promoting substances like Indole acetic acid (IAA), Gibberellins, Auxin, Vitamins, and hormones is well recognized in microorganisms. The capacity of 316 cultures for the synthesis of thiamin, biotin, riboflavin, and vitamin B_{12} is examined It is found that riboflavin is produced by a large number of isolates, followed by thiamin and vitamin B_{12}.[28] Maximum production of auxin by *P.straita, B.polymyxa*, and the least auxin activity was recorded with *A.awamori*. The gibberellins activity is recorded more in *B.polymyxa* where a slight inhibitory effect was observed.[29]

In this study, it was found that *A.awamori, Ps. straita a*nd *T.viridae* are more efficient in solubilization of RP and TCP also, whereas *A.niger, A.oryzae, B.megaterium* efficiently solubilized TCP but not Rock Phosphate. In contrast to this, RP was solubilized very efficiently by *B.polymyxa* as comparing with TCP.

The endophytic *Pseudomonas* strains L111, L228, and L321 have shown good phosphate solubilization. *Pseudomonas fluorescens* L321 also enhanced plant growth promotion of *P. sativum L. plants* under phosphate limiting environments and in particular.[30] Among the phosphate solubilizing microorganisms that facilitate plant growth and development under several stresses, Pseudomonas sp. enhanced plant growth in salt stress.[31] The *Pseudomonas aeruginosa* isolates among the PSB isolated from the chili rhizosphere interestingly showed the presence of various potential plant growth-promoting properties including indole acetic acid and siderophore production, and an increase in available P. This has confirmed the growth-promoting potential of the isolates to develop as biofertilizers.[32]

In the present work, germination study showed excellent results with *Ps. straita* in both radical and plumule in 1% and 2% concentration of culture. This was followed by a 1% concentration of *B.polymyxa* culture. Germination of moth gram seeds in association with *P.straita* showed the length of radical and plumule in 1% concentration is about 28 mm and 45 mm respectively, and in 2% it is 32 mm and 40 mm. It is more consistent and more satisfactory than other used efficient PSMs.

The supplementary property of phyto-hormone secretion by phosphate-dissolving microorganisms can increase crop growth. *Pseudomonas striata* assessed for phytate mineralization and solubilization of tricalcium, rock, ferric, and aluminum phosphate exhibited very good potential as phosphobacteria. Chromatographic analysis of cell-free culture filtrate exposed the presence of tartaric acid, malic acid, citric acid, succinic, and gluconic acid. Tartaric acid was effective in the solubilization of TCP. The extracellular phytase (43.05 EU ml^{-1}) was synthesized in a phytase-specific broth medium by the strain of *Pseudomonas striata*. A higher level of indoleacetic acid (15.59 μg ml $^{-1}$) was noted in the absence of tryptophan than in its presence.[33] The effects of seed soaking with *Bacillus subtilis* strain GB03 suspension culture and its volatile organic compounds on seed germination of a plant *Codonopsis pilosula* which is a traditional Chinese herbal crop were investigated. The results showed that the seed soaking with GB03 suspension culture and its volatile organic compounds has improved seed germination, mainly more effective on seed germination potency. GB03 expressively enhanced shoot and root length, branching, plant biomass (whole plant fresh and dry weight), leaf area, and chlorophyll content in *C. pilosula* seedlings after 0, 20, 40, and 60 days of soil inoculation. Besides the decreased intercellular CO2 concentration, GB03 significantly enhanced transpiration rate, stoma conductance, and net photosynthetic rate.[34]

In the case of *B.polymyxa,* 2% concentration showed radical - 32 mm and plumule - 22 mm, but in 1% concentration, it was observed more than 2% viz. 50 mm - radical and 25 mm- plumule. This fact is also observed in other organisms. There was only the difference between the length of radical and plumule concerning the individual strain. The length of radical and plumule in control is recorded 14 mm and 03 mm respectively. The results for *A.niger, A.awamori, T. viridae.* The lowest effect was observed with *T.viridae,* (Plumule - 11 mm in 1%) and *A.awamori* (Radical -22 mm at 2%) on treatment.

Yadav *et. al.* (2011) have observed that the single inoculation of A. *niger* and *P. citrinum* did not show significant shoot length between each other but a significant increase over control.[35] The various growth parameters were improved significantly and the ability of *Aspergillus awamori* was restored to colonize maize roots due to the application of IAA.[36] The majority of the mycotoxins such as aflatoxins, citrinin, patulin, penicillic acid, tenuazonic acid, ochratoxin A, cytochalasins, deoxynivalenol, fumonisins, fusarin C, fusaric acid, and zearalenone produced by three fungal genera: Aspergillus, Penicillium, and Fusarium are considered the types that most contaminate cereal grain. The mycotoxins primarily affect the seed quality, germination, viability, seedling vigor, growth of root, and coleoptile.[37] In a similar study carried out by Garuba *et. al.* (2014), it is found that the culture filtrates of both *A. niger* and *P. chrysogenum* affected not only the percentage of seed germination but also the morphology and anatomy of maize seedlings. It

adversely affected the epidermal cells and stomata. The percentage of germination of maize seeds soaked in *A. niger* filtrate (65.33%) was significantly different from the seeds soaked in *P. chrysogenum* filtrate (79.6%) and the control experiment (100%). The vigor indices of experimental plants were expressively different from each other at $p \leq 0.05$.[38]

Trichoderma spp. are capable of producing secondary metabolites like non-ribosomal peptides, terpenoids, pyrones, indolic derived compounds, and fungal auxin-like compounds, which can trigger systemic resistance and plant nutrient uptake and causes increased root and shoot growth.[39] Most classical plant hormones are synthesized by pathogenic and symbiotic fungi. The phenomenon of molecules favors the invasion of plant tissues and the development of fungi inside plant tissues is not clear yet. There is a possibility of two modes of action of these molecules synthesized by fungi (i) positive or negative bias in plant processes, to favour invasion and nutrient uptake; and (ii) to act as signals for the fungi themselves to involve proper developmental and physiological processes adapted to their environment. The abscisic acid, gibberellic acid, and ethylene produced by fungi may contribute to pathogenicity. It is noted that auxin and cytokinins might be essential positive regulators for virulence.[40] The fungal secretions showed pathogenic effects on plant growth. The germination rate of wheat grains irrigated with the filtrate of *A. niger* and Rhizopus sp. was 20% and 80% respectively, compared with 100% of the control grains, which were irrigated with water. The culture filtrates of A. niger and Rhizopus sp. affected the percentage of grains germination and the morphology of wheat seedlings. It adversely affected the length of the radicles and coleoptiles.[41]

The maximum shoot length and root length of green gram were significantly superior over all other strains and SSP control. Most of the PSB strains were able to improve the root and shoot growth of green gram plants compared to SSP control and RP control.[42] pH range of *Pseudomonous spp.* was initially at 6.8 and finally decreased to 4.63 then produce more Indole acetic acid. The pH range of *Aspergillus niger* was initially at 6.8 and finally reduced to 4.29 and produce more Indole acetic acid in Fungi. Then finally the IAA was observed in thin layer Chromatography. There is increasing evidence that phosphobacteria improve plant growth due to biosynthesis of plant growth substances rather than their action in releasing phosphorous.[43] The results of seed germination in the present study are in agreement with the above findings.

Phosphate solubilizing microbes promise a perpetual source of phosphate to the crops. It is eco-friendly and cost-effective agro-technology to increase crop yield. Hence, research in this field for developing this technology and to minimize the use of chemical fertilizers, and make use of biofertilizers is beneficial.[44]

Therefore it can be stated based on the results obtained in the present study that the *P.straita* and other efficient PSMs can be used individually or as a consortium to develop a vigorous biofertilizer for the various crops after further research.

5. Source of Funding

None.

6. Conflict of Interest

The authors declare that there is no conflict of interest.

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