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EFFECT OF SINGLE AND DUAL INOCULATIONS OF ARBUSCULAR MYCORRHIZAS ON SOIL PHOSPHATASE, GROWTH, AND ROOT COLONIZATION OF TOMATO

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ABSTRACT

Soil phosphatase, growth and root colonization of tomato treated with two strains of arbuscular mycorrhizal fungi (AMF) - Rhizophagus irregularis and Rhizophagus intraradices in single and dual inoculations were investigated. The experiment was set up in a completely randomized design with four treatment groups: R. irregularis (R.irr), R. intraradices (R.int), R.irr+R.int, and uninoculated (control), for six weeks in a greenhouse, after which the plants were harvested. The data collected on soil acid phosphatase, root colonization, growth indices and shoot phosphorus (P) concentrations were analyzed using one-way ANOVA at 95% probability level. Soil acid phosphatase and shoot P concentrations were significantly ($p < 0.05$) increased by AMF inoculation, with higher phosphatase values in dual inoculation (0.43 µg g^{-1}) compared to the single inoculation of R.irr (0.32 µg g^{-1}) and R.int (0.34 µg g^{-1}), and the control (0.14 µg g^{-1}). Compared to the control, R.irr and R.irr+R.int significantly increased the shoot fresh weights and total dry weights, while plant lengths and number of leaves were highest in dual inoculation. However, the intensity of mycorrhizal colonization (41.32%, 20.57% and 38.18%) recorded for R.irr, R.int, and R.irr+R.int, respectively, and the corresponding growth responses (30.99%, 24.56% and 27.48%), were not significantly ($p > 0.05$) different between single and dual inoculated plants. Although a significant synergistic effect on host colonization and biomass indices was not obtained, dual inoculation of R. irregularis and R. intraradices induced more soil phosphatase production in tomato rhizosphere than single inoculation.

Key words: growth-promotion, mixed inoculum, AMF consortium, synergism, phosphorus

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a popular crop cultivated in many parts of the world and one of the most widely consumed vegetables (Dorais *et al*., 2008). It is a regular feature of Nigerian diet and is cultivated more in the Northern and Middle-belt of Nigeria than in the Southern part of the country (Olarenwaju, 2017). In line with its increasing population, Nigeria's tomato needs have been increasing, contributing to steadily higher prices over the years, and the need to improve its country-wide production. Tomato production in Nigeria relies substantially on irrigation and chemical fertilizer inputs, and some challenges facing the crop production side of its value-chain includes pests, diseases, and soil fertility issues, amongst others (Ugonna *et al*., 2015; Ibrahim *et al*., 2020). To minimize the need for increased use of chemical fertilizers by growers, the efficient utilization of biofertilizers for improving the crop's nutrition and productivity is a cheap and ecofriendly option to be explored. This is in addition to the potential improvements in nutritional value of tomato fruits due to the action of competent bioinoculants (Hart *et al*., 2015; Ibiang and Sakamoto, 2022) and their improved tolerance to diverse environmental stresses (Volpe *et al*., 2018; Hashem *et al*., 2021).

Phosphorus (P) is a major element necessary for plant growth, but may easily be a growth-limiting nutrient due to its generally low phytoavailability in soils (Alewell *et al*., 2020; Chukwuma *et al*., 2024). It is subject to loss due to erosion and leaching in soils (McGroddy *et al*., 2008) and even when soils contain sufficiently high levels of total P, up to 85-90% of this is precipitated and immobilized with other elements in soil, such as iron (Fe), aluminum (Al), and calcium (Ca), thereby greatly reducing the amount available for plant uptake (Van der Bom *et al*., 2019). Some beneficial microbial inoculants can ameliorate some of these constraints and increase P availability and uptake by plants. Symbionts like arbuscular mycorrhizal fungi (AMF) increase plant P nutrition via several mecha-nisms including modulation of soil pH, release of organic acids and enzymes such as phosphatase, increasing root lengths and surface area, amongst others (Lee *et al*., 2014; Berruti *et al*., 2016; Rawat *et al*., 2021). With respect to P nutrition, phosphatases are significant enzymes of agronomic importance in soils, as they hydrolyze organic P compounds into inorganic forms that are more readily available for plants to absorb (Manyapu *et al*., 2022).

The AMF are root colonizers that are known to enhance plant growth and fitness, in addition to other physiological effects. Their abundance and

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diversity benefits soil health and fertility in general, making them potentially useful in plant production and soil management (Smith and Read, 2008; Olawuyi *et al*., 2022). In tomato, previous reports have indicated the benefits of AMF inoculation to include increased plant growth, nutrient uptake, and stress tolerance. However, the soil condition, AMF species identity and inoculation strategy (single vs mixed species) are important factors influencing the reported outcomes (Ibiang *et al*., 2018; Chandrasekaran *et al*., 2021). In the quest to maximize the beneficial outcomes on plant growth and nutrient uptake by AMF, the use of inoculum consisting of two or more species has been touted by some researchers for its potentially wider spectrum of advantages to hosts (Koide, 2000; Wagg *et al*., 2011; Cao *et al*., 2021). One reason for this is the belief that mixed species inoculum could be more resilient in the environment and more efficient on the host compared to single species inoculation (Trejo-Aguilar and Banuelos, 2020). Although individual AMF species may differ in the responses they induce on colonized plants, it is not easily known whether they will exhibit competitive, additive, or synergistic interaction during co-inoculation (Wagg *et al*., 2011; Boyer *et al*., 2015).

On the other hand, there is also the view that maximum benefit to host can be achieved by single inoculation of an effective AMF fungus, compared to mixed species inoculation (Edathil *et al*., 1996; Hart *et al*., 2013; Malicka *et al*., 2021). Therefore, evaluating the tendencies between two or more target AMF species can provide information for determining the strategy of their deployment and use, especially where additive or synergistic outcomes on host or soil indices are indicated. This study examined the effect of single and dual inoculation of two species of AMF on the soil phosphatase, growth, root colonization as well as P nutrition of tomato grown in Calabar, Nigeria. The two species of AMF tested in this study (*Rhizophagus irregularis* and *Rhizophagus intraradices*) are popular and fairly widespread, but to the best of our knowledge, this is the first time they were tested for possible synergic effects on tomato grown in Calabar.

MATERIALS AND METHODS

Soil and Seedling Preparation

The soil utilized for this study was sandy-loam topsoil (up to 20 cm deep) collected from the experimental farm of the Department of Genetics and Biotechnology, University of Calabar, Nigeria. The soils were bulked and sieved using a soil sieve (2 mm) to remove stones and other debris and analyzed for pH (6.6), EC (1.3 mS m^{-1}), organic carbon (0.25%), total N (0.15%), available P (0.68 mg kg^{-1}) and autoclaved (at 121°C for 60 min.) before use. The tomato, *Solanum lycopersicum* (L.) (cv. Rodeo) was used in this study. The seeds were sterilized in 70% ethanol for 5 min and 3.33% sodium hypochlorite solution for 15 min. then rinsed repeatedly in sterile distilled water before seeding in vermiculite. The seedlings were raised for 21 days on vermiculite moistened with modified Hoagland solution (to supply nutrients) twice a week and then transplanted to potted soils in the greenhouse.

AMF Inoculation

The AMF inoculants used in this study were *Rhizophagus irregularis* DAOM197198 and *Rhizophagus intraradices* 15S-1 both obtained from the Lab of Plant Nutrition, Chiba University, Japan, and reproduced locally in separate pots using soybean plants. These AMF were selected based on their widespread availability and previous testing on tomatoes which demonstrated improved host growth and stress tolerance (Ibiang *et al*., 2018; Ibiang *et al*., 2020; Chandrasekaran *et al*., 2021). The inoculation was performed at the time of seedling transplant from vermiculite to potted soils. The inoculum for each mycorrhizal species consisted of soil bearing AMF propagules - hyphae and mycorrhizal root fragments with a few intraradical spores - applied (5 g pot^{-1}) manually in the middle of potted soil just prior to seedling transplant. The pot consisted of labelled 16 cm × 24 cm black polythene bags, filled with 3 kg of soil. A hole (2 cm deep) was made in the center into which the AMF inoculum was placed before a seedling was inserted and the hole covered. For single inoculation, individual inoculum was applied at the quantity stated above, but for dual inoculation, a 50:50 (w w^{-1}) mixed inoculum of both species (Boyer *et al*., 2015) was applied, while uninoculated control pots received no inoculum.

Experimental Setup

The experiment was laid out in a completely randomized design (CRD) with four treatment groups; uninoculated control (C), *Rhizophagus irregularis* (R.irr), (*Rhizophagus intraradices* (R.int), and dual inoculation (R.irr+R.int), each with five replicates, giving a total of twenty pots maintained in the greenhouse of the Department of Genetics and Biotechnology, University of Calabar, for six weeks. All plants were routinely supplied with borehole water and modified Hoagland solution given at 50 mL pot^{-1} on the day of transplant and once a week for the first four weeks, then twice a week for the remaining two weeks until harvest.

Plant Harvest and Measurement of Growth Indices

After counting the number of leaves, each plant was wholly harvested by carefully emptying the soil from the pots onto labeled polythene bags, washing soil loosely attached to roots in a bucket of water and rinsing in distilled water. The whole plant was then cut into roots and shoots, and their lengths and fresh weights were measured using a measuring tape and an electronic weighing balance (Golden-Mettler USA). A portion (0.5 g) of the roots of each plant was

collected for the determination of mycorrhizal colonization and stored in tubes containing a solution of formaldehyde: acetic acid: alcohol (1:1:18 v/v/v), before drying all fresh biomass in the oven at 80°C for 48 h. The mycorrhizal growth response was estimated using total plant dry weights as:

growth response (%) =
$(DW_{(AM)} - \text{mean } DW_{(non\text{-}AM)})/(\text{mean } DW_{(non\text{-}AM)}) \times 100$
(Watts-Williams and Cavagnaro, 2012).

Arbuscular Mycorrhizal Colonization

Mycorrhizal colonization was assessed in the root subsamples of all plants using the trypan blue staining technique previously described by Rajapakse and Miller (1994) after clearing the roots in 10% KOH solution. Thirty root sections of 1 cm lengths were mounted on a slide and observed in a light microscope under ×10 lens for mycorrhizal scoring according to Trouvelot *et al*. (1986).

Plant Phosphorus Concentration

Phosphorus concentration was determined using the vanadomolybdate method as described by Tandon *et al*. (1968). Dry finely ground shoot samples were digested in 0.6 mol L^{-1} HCl acid and reacted with vanadomolybdate acid solution. Potassium dihydrogen phosphate (KH_2PO_4) was used as the standard and samples were kept for 30 min after which their absorbance was measured in a spectrophotometer (U-1800 Hitachi High-Tech Corp) at 420 nm.

Soil pH and Phosphatase Determination

Soil acid phosphatase activity was determined using p-nitrophenyl phosphate (PNP) according to the method of Tabatabai and Bremner (1969). About an hour after roots were recovered, 1 g of soil obtained from each pot was mixed with 4 mL of modified universal buffer (MUB), toluene (0.25 mL) and PNP (1 mL) and swirled before corking with stoppers and placing in an incubator for 1 h at 37°C. Afterwards, 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH were added, and it was swirled to mix before filtering and measurement in a spectrophotometer at 420 nm, with phosphatase activity expressed as p-nitrophenol released per gram of soil (Hu *et al*., 2015). For soil pH, 10 g of air-dried soil was mixed with 50 mL of distilled water, then vortexed twice at maximum speed for 30 secs, allowed to stand for 1 h, and vortexed again before measuring the pH.

Statistical Analysis

The data collected were processed statistically by one-way analysis of variance (STATCEL ver. 4), with significance level set at $p < 0.05$, after Levene homogeneity test, and the differences between treatment group means were established based on Tukey-Kramer post-hoc tests.

RESULTS

The growth of tomato in the greenhouse was generally observed to be better in the inoculated groups compared to control (Figure 1). At harvest, shoot lengths (Figure 2a) were generally higher in the AMF inoculated groups, with dual inoculation having the highest values (34.56 cm) and the control having the lowest (26.62 cm) but there were no significant differences ($p>0.05$) between the groups. Root lengths (Figure 2b) were not significantly different but the highest values were observed in dual inoculation (17.7 cm) and the lowest in single *R. intraradices* (13.92 cm) treatment. The number of leaves per plant (Fig. 2c) was significantly different ($p < 0.05$) with higher values in dual inoculation (9.6) than in the control (6.8). The leaf numbers in singly inoculated groups of *R. irregularis* (8.8) and *R. intraradices* (7.6) were not different from control and dual inoculation. Root fresh weight (Figure 2d) showed significant differences with higher values in *R. irregularis* (5.1 g) compared to control (3.2 g), while *R. intraradices* (3.6 g) and dual inoculation (3.8 g) were not different. Shoot fresh weight (Figure 2e) also showed significant differences with higher values in *R. irregularis* (12.6 g) and dual inoculation (13.2 g) compared to control (9.0 g), while the inoculated groups were not different. Total dry weight (Figure 2f) showed significant differences with higher values in *R. irregularis* (4.48 g) and dual inoculation (4.36 g) compared to the control treatment (3.42 g), which had the lowest.

The mycorrhizal growth response (Table 1) indicated biomass increase of 24 - 30% due to AMF inoculation but there were no differences between the single and dual inoculation. The frequency and intensity of root colonization (Table 1) indicated comparable levels of colonization for both AMF species in single inoculation as well as the dual inoculation, with no significant differences between the groups. Nevertheless, *R. irregularis* showed the

Figure 1: Tomato plants inoculated with single and dual species of arbuscular mycorrhiza after 4 weeks of cultivation in the greenhouse
From left to right; Control, *R. irregularis*, *R. intraradices*, and *R. irregularis* +*R. intraradices*

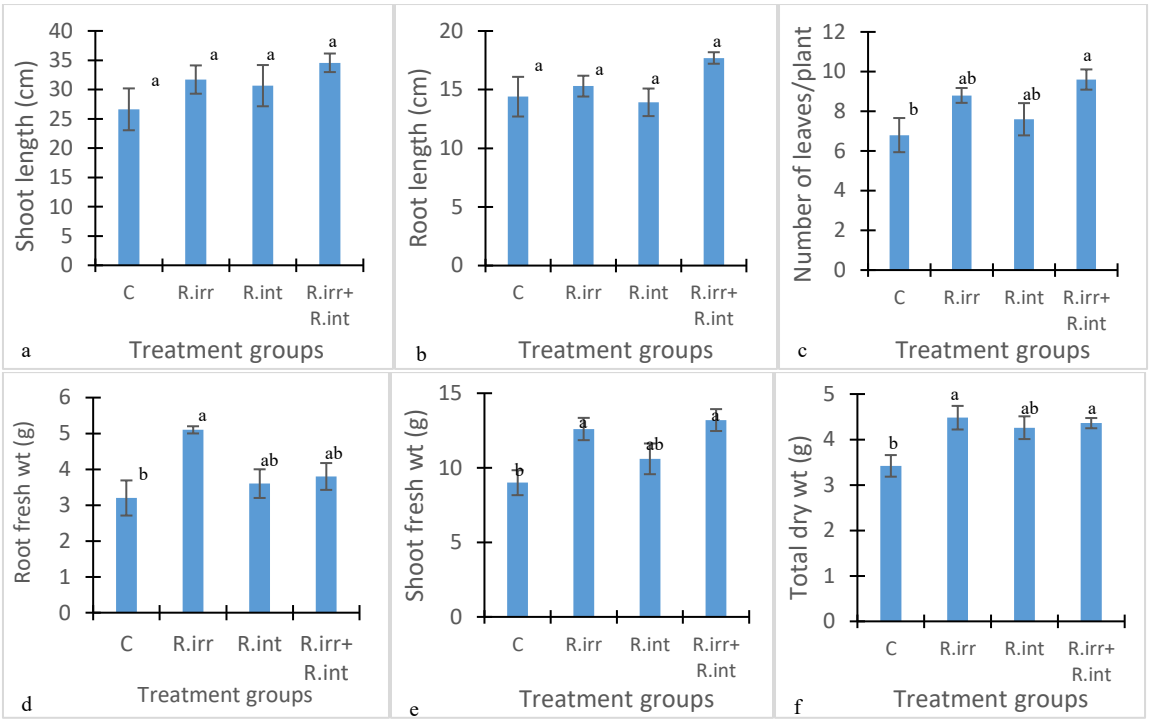

Figure 2: Plant growth indices including shoot length (a), root length (b), number of leaves per plant (c), root fresh weight (d), shoot fresh weight (e), and total dry weight of tomato (f) inoculated with single and dual species of arbuscular mycorrhiza. Values are mean±SE ($n = 5$). [abc]Superscripts indicate differences based on Tukey-Kramer post hoc tests. C - Control), R irr - *R. irregularis*, R. int - *R. intraradices*), R.irr+R.int - *R. irregularis* + *R. intraradices*

highest intensity of colonization at 41.32% while colonization was not observed in the control plants. The shoot P concentration (Table 1) showed significant differences between control (4.70 mg g^{-1} dw) and AMF inoculated plants where *R. intraradices* (6.70 mg g^{-1} dw) had the highest values. Soil phosphatase (Table 1) showed significant differences between the control (0.14 µg g^{-1}) and AMF inoculated groups, with the values in dual inoculation (0.43 µg g^{-1}) being higher than single inoculation. Soil pH (Table 1) showed significant differences between the groups with higher values in control (6.47) and *R. irregularis* (6.49) than in *R. intraradices* (6.19) and dual inoculation (6.26).

DISCUSSION

Previous reports indicating increases in tomato growth due to *R. irregularis* and *R. intraradices* points to physiological mechanisms by which they promote growth to include enhanced P and nitrogen nutrition (Ruiz-Lozano *et al*., 2016; Xu *et al*., 2018), chlorophyll biosynthesis (Ibiang *et al*., 2020), and micronutrient homeostasis, e.g., Cu (Ibiang *et al*., 2018), amongst others. In this study, the increase in tomato growth indices due to AMF reflect in the positive growth responses observed in the inoculated plants. However, the largely comparable values between the single and dual inoculations imply that there was not a substantial additive or synergic benefit to host due to *R. irregularis* and *R. intraradices* co-inoculation. The host growth results also indicate the absence of competitive tendency between the two inoculated strains which may result in poorer plant growth in mixed-species inoculation compared to single (Malicka *et al*., 2021). Other studies which report similar improvements in host growth between single and dual inoculations exist, such as Boyer *et al*. (2015) who observed that single species inoculation of *Funneliformis mosseae* and *Funneliformis geosporus* gave similar benefits to the host as did the mixed-species inoculation. Hart *et al*. (2015) also reported that dual inoculation of *R. irregularis* and *F. mosseae* did not significantly increase shoot dry weights of tomato more than single inoculation.

The species/strain of AMF in a mixed-species consortium, alongside the growth conditions, may play a role in growth response. Under abiotic stress conditions, cases where dual inoculation of AMF led

Table 1: Root colonization, growth response and phosphorus nutrition indices of tomato inoculated with single and dual species of arbuscular mycorrhizal fungi

	Control	*R. irr*	*R. int*	*R. irr* + *R. int*	*P*-value
Frequency of colonization (%)	-	96.66 [a] ±1.49	95.33 [a]±2.49	98.00 [a]±1.33	0.6065 ns
Intensity of colonization (%)	-	41.32 [a]±7.40	20.57 [a]±6.15	38.18 [a]±4.30	0.0685 ns
Growth response (%)	-	30.99 [a]±7.59	24.56 [a]±7.31	27.48 [a]±3.48	0.7788 ns
Shoot P (mg g^{-1} dry wt.)	4.72 [b] ±0.21	6.20 [a]±0.08	6.70 [a]±0.22	6.57 [a]±0.15	2.5E-06***
Soil phosphatase (µg g^{-1} soil)	0.14 [c]±0.01	0.32 [b]±0.01	0.34 [b]±0.01	0.43 [a]±0.01	3E-09***
Soil pH	6.47 [a]±0.02	6.49 [a]±0.02	6.19 [b]±0.02	6.26 [b]±0.04	5.1E-06***

Values are Mean ± SE (n=5), abc - letters denoting differences on the basis of Tukey-Kramer tests. P-values are based on one-way ANOVA. C (control), R.irr - R.irregularis, R.int - R.intraradices, R.irr+R.int - R.irregularis+R.intraradices

to significantly better host growth than single inoculation have been reported. For example, Parvin *et al*. (2020) reported that dual inoculation of *Acaulospora laevis* and *Gigaspora margarita* increased rice growth under salt stress better than either single inoculation. Previous reports in tomato using the same AMF inoculants indicates similar levels of colonization (Ibiang *et al*., 2018, 2020) as was observed here. Similar frequency and intensity of mycorrhizal colonization between single and dual inoculated plants indicates no change in the root colonization stemming from the interaction between both symbionts. Thus, it can be assumed that neither AMF was subdued during the co-colonization and each interacted with the host unencumbered by the other, in the dual inoculated plants (Boyer *et al*., 2015). Plant roots and phosphate-solubilizing micro-organisms secrete organic acids and non-specific acid phosphatases (NSAPs) into the rhizosphere to increase phosphorus availability in soils for root uptake (Wasaki *et al*., 2013; Rawat *et al*., 2021). Mycorrhizas are also known to increase phosphatase activity in rhizosphere (Gyaneshwar *et al*., 2002), as the extraradical hyphae of AMF extends beyond the immediate vicinity of the roots and mine P and other nutrients from soil. Sharda *et al*. (2010) reported that AMF inoculation increased the phosphatase activity in the rhizosphere of *Carica papaya* and increased P uptake, while Hu *et al*. (2015) reported that increased soil phosphatase activity coincided with higher external mycelium lengths of AMF in the soil.

Aside modulating phosphatase, AMF can alter rhizosphere soil pH by hyphal release of entities such as H^+ and organic acids, as well as modulation of host root exudation, which modify solubility and availability of forms of P in the soil (Gyaneshwar *et al*., 2002). The higher soil phosphatase in AMF inoculated groups than in the control, and the corresponding increases in shoot P concentrations is in line with these reports, as with the conclusions drawn by Qin *et al*. (2019) in their study. Furthermore, higher phosphatase values in dual inoculation compared to single, points to an enhanced effect of the AMF consortium. This indicates an aspect of soil health that is impacted by AMF species abundance and diversity, in line with their ecosystem services (Hu *et al*., 2015; Oehl *et al*., 2017). Since organic phosphates constitute 20-30% of total phosphorus in soils, their dissolution by acid phosphatases should add to the pool of soluble P accessible for root uptake and enhance P nutrition (Rawat *et al*., 2021). However, higher soil phosphatase in dual inoculation did not translate into higher phosphorus concentrations, compared to single inoculation; implying that single inoculation of either AMF was as effective as dual inoculation in increasing P nutrition in tomato hosts. Since the soil used was acidic, it was assumed that there were more Fe-P and Al-P complexes than Ca-P (Van der Boom *et al*., 2019), thus, the changes in soil pH detected in *R. intraradices* and dual inoculation likely contributed to enhancement of phosphorus solubility and uptake in these inoculated groups.

CONCLUSION

Single and dual inoculation of *Rhizophagus irregularis* and *Rhizophagus intraradices* improved phosphorus nutrition and tomato biomass production by 24-30%. Although a significant synergistic effect on host colonization and biomass indices was not obtained, dual inoculation of *R. irregularis* and *R. intraradices* induced more soil phosphatase production in tomato rhizosphere than single inoculation, indicating a better improvement of the soil fertility parameter by the AMF consortium in tomato rhizosphere.

DECLARATION OF COMPETING INTERESTS

None declared.

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