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# Synergistic interaction of *Rhizobium leguminosarum* bv. *viciae* and arbuscular mycorrhizal fungi as a plant growth promoting biofertilizers for faba bean (*Vicia faba* L.) in alkaline soil☆

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ABSTRACT

Egyptian soils are generally characterized by slightly alkaline to alkaline pH values (7.5–8.7) which are mainly due to its dry environment. In arid and semi-arid regions, salts are less concentrated and sodium dominates in carbonate and bicarbonate forms, which enhance the formation of alkaline soils. Alkaline soils have fertility problems due to poor physical properties which adversely affect the growth and the yield of crops. Therefore, this study was devoted to investigating the synergistic interaction of *Rhizobium* and arbuscular mycorrhizal fungi for improving growth of faba bean grown in alkaline soil. A total of 20 rhizobial isolates and 4 species of arbuscular mycorrhizal fungi (AMF) were isolated. The rhizobial isolates were investigated for their ability to grow under alkaline stress. Out of 20 isolates 3 isolates were selected as tolerant isolates. These 3 rhizobial isolates were identified on the bases of the sequences of the gene encoding 16S rRNA and designated as *Rhizobium* sp. Egypt 16 (HM622137), *Rhizobium* sp. Egypt 27 (HM622138) and *Rhizobium leguminosarum* bv. *viciae* STDF-Egypt 19 (HM587713). The best alkaline tolerant was *R. leguminosarum* bv. *viciae* STDF-Egypt 19 (HM587713). The effect of *R. leguminosarum* bv. *viciae* STDF-Egypt 19 and mixture of AMF (*Acaulospora laevis*, *Glomus geosporum*, *Glomus mosseae* and *Scutellospora armeniaca*) both individually and in combination on nodulation, nitrogen fixation and growth of *Vicia faba* under alkalinity stress were assessed. A significant increase over control in number and mass of nodules, nitrogenase activity, leghaemoglobin content of nodule, mycorrhizal colonization, dry mass of root and shoot was recorded in dual inoculated plants than plants with individual inoculation. The enhancement of nitrogen fixation of faba bean could be attributed to AMF facilitating the mobilization of certain elements such as P, Fe, K and other minerals that involve in synthesis of nitrogenase and leghaemoglobin. Thus it is clear that the dual inoculation with *Rhizobium* and AMF biofertilizer is more effective for promoting growth of faba bean grown in alkaline soils than the individual treatment, reflecting the existence of synergistic relationships among the inoculants.



## 1. Introduction

The United Nations Food and Agriculture Organization (FAO) estimates that the total demands for agricultural products will be 60% higher in 2030 than present time and more than 85% of this additional demand will come from developing countries. For over half a century, the world has relied on increasing crop yields to supply an ever increasing demand for food. Soil plays a major role in determining the sustainable productivity of an agro-ecosystem. The sustainable productivity of a soil mainly depends upon its ability to supply essential nutrients to the growing plants. The deficiency of micronutrients has become major constraint to productivity, stability and sustainability of soils (Bell and Dell 2008). Soil alkalinity is one of the most common problems in arid and semi-arid regions and characterized by high pH ranged between 7.5 and 8.7 (Perry and Perry 1989; Clark 1996). Arid land in Egypt represents 97% of the total area, characterized by high temperature, low relative humidity, high rate of evaporation, and little rainfall, leading to degraded soils. The problem of soil alkalinization due to $NaHCO_3$ and $Na_2CO_3$ may be more severe than that of soil salinization caused by neutral salts, like NaCl and $Na_2SO_4$ (Shi and Yin 1993; Tang and Turner 1999; Shi and Sheng 2005). Egypt soils are generally characterized by slightly alkaline to alkaline pH values which are mainly due to its dry environment. As soil salinization and alkalinization frequently co-occur, the conditions in naturally

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salinized and alkalinized soils are very complex; the total salt contents, their composition and the proportion of neutral to alkaline salts may vary in different soils (Islam et al. 2011). Alkaline soils are characterized by poor availability of phosphorus and micronutrient transition metals (Fe, Cu, Mn, and Zn); also alkali stress usually involves a combination of stresses, osmotic, ion-induced injury and high pH (Lynch and St Clair 2004; Munns 2002; Chen et al. 2011).

Currently, there is an emerging demand to decrease dependence on chemical fertilizers and increase the sustainability of agriculture. Wang et al. (2011) reported that during the past 50 years, the widespread use of chemical fertilizers to supply N and P has had a substantial impact on food production, and has become a major input in crop production around the world. Today, only 30–50% of applied N fertilizer and 10–45% of P fertilizer are taken up by crops (Adesemoye and Kloeppe 2009).

One of the most important factors in the generation of high yields from crop plants is nitrogen fertilizer. That is why farmers are applying high amounts of the fertilizers which is very costly and make the environment hazardous especially when used discriminately. In addition, more than 50% of the applied of nitrogen and phosphorus fertilizers are somehow lost through different processes which not only represent a cash loss to the farmers but also consequently polluted the environment (Ladha et al. 1998). Scientists all over the world are facing this alarming situation and they are trying to overcome this condition by exploring alternative sources which are cost effective and save the environment. Biofertilizer, an alternative source of N and P fertilizer, especially rhizobia and AMF in legume symbiosis, is a promising technology. The capacity of plant to acquire and utilize nutrients has encouraged researchers to study how nutrient efficiency is influenced by both nutrient absorption in roots by plant growth promoting rhizobacteria and utilization in plants (Sepehr et al. 2009). In rhizosphere, some beneficial bacteria and fungi are present and they improve plant performance under different environmental conditions. They play a key role in natural ecosystems and influence plant productivity, plant nutrition and improved inhibition of fungal plant pathogens (Demir and Akkopru 2007; Wehner et al. 2010; Abohatem et al. 2011).

Rhizobia are the most studied PGPR for their potential to fix $N_2$ in the leguminous plants. Rhizobia is the common name given to a group of small, rod-shaped, Gram-negative bacteria that collectively have the ability to produce nodules on the roots of leguminous plants and belong to the family Rhizobiaceae, which are part of the α-proteobacteria. The symbiosis between a leguminous plant and its microsymbiont is characterized by a multistep signal exchange process.

During growth in the rhizosphere of a host plant, rhizobia sense compounds such as flavonoids and betaines secreted by the host root and respond by inducing nod genes (Laeremans and Vanderleyden 1998; Abd-Alla 1999a, 2011; Gage 2004). The nod genes encode approximately 25 proteins required for the bacterial synthesis and export of Nod factor. Nod factor is a lipooligosaccharide signal consisting of a chitin backbone, four to five N-acetylglucosamine units in length, with a lipid attached to the nonreducing end and host-specific modifications on the backbone. Nod factor initiates many of the developmental changes seen in the host plant early in the nodulation process, including root hair deformation, membrane depolarization, intracellular calcium oscillations, and the initiation of cell division in the root cortex, which establishes a meristem and nodule primordium.

The symbiosis between legumes-*Rhizobium* and arbuscular mycorrhizal fungi (AMF) is one of the important ecological mutualisms (Remy et al. 1994). This symbiosis between roots and the fungal phylum of Glomeromycota originated around 450 million years ago and may have been a key event that enabled plants to colonize emerging landscapes (Remy et al. 1994). AMF are associated with the roots of over 80% of terrestrial plant families (van der Heijden et al. 1998). AMF plays a key role in the regulation of ionome and membrane transport proteins that control the ion homeostasis of the host plants (Ramos et al. 2011; Song and Kong 2012). AMF is known to exist in stressed soil, and participates in the plant growth and development, and also improves the plant tolerance against biotic and abiotic stress (Abd-Alla et al. 2000; Berta et al. 2005; Abdel-Fattah et al. 2010) by regulating the physiological and biochemical process of plants (Evelin et al. 2009; Fernanda et al. 2012).

The majority of legumes form symbiotic associations with both phosphorus-acquiring AMF and nitrogen (N)-fixing rhizobia in tripartite relationship (Barea et al. 2005; Meghvansi et al. 2008; Kaschuk et al. 2010). The micro-symbionts in both associations are benefited by photoassimilates from the plant. The macrosymbiont obtains fixed nitrogen in case of bacterial symbiosis of root nodules (Crespi and Galvez 2000) and immobile nutrients especially phosphate in case of AM symbiosis (Miransari et al. 2009). The legumes and their association with rhizobia and AMF in the broad sense have always been important agronomically. Although, there are many studies on the interactions between AM and bacteria, the underlying mechanisms behind these associations are not yet well understood (Chalk et al. 2006).

The utilization of plant growth-promoting micro-organisms is useful in strategies to facilitate plant growth in saline stressed soils (Tank and Saraf 2010). Plant growth-promoting bacteria are soil and rhizosphere bacteria that can be of benefit to plant growth by several different mechanisms such as asymbiotic $N_2$ fixation, ammonia production, solubilization of mineral phosphate and other essential nutrients, production of plant hormones, and control of phytopathogenic microorganisms (Tank and Saraf 2003). In addition, PGPR can protect plants from deleterious effects of environmental stress like flooding, drought, salinity and heavy metals. There is, however, no information available about the effect of combined inoculation with *Rhizobium leguminosarum* and mixed inoculum of AMF to alleviate the noxious effect of alkalinity stress on nodulation, nitrogen fixation and growth of faba bean.

The objective of this study was to investigate interactions between native *Rhizobium* and mixture of AMF to provide a clear understanding of this tripartite association and its impact on nodule formation, AM colonization, nitrogen fixation and plant growth of faba bean plants grown under alkalinity stress.

## 2. Methods

### 2.1. Isolation of rhizobial isolates

In the current work three representative sites were chosen from Assiut, Egypt (27.179032°N, 31.185567°E); the three sites were Mosha village, El-Zawya village and Doronka village. The strains of rhizobia were isolated from root nodules of *Vicia faba*. The isolates were grown on yeast extract mannitol agar (YEMA) medium and incubated at 28 °C (Vincent 1970) on an orbital shaker at 120 rev per min for three days. The growth response of bacterial isolates at pH 9 was assessed by following the OD at 600 nm using Unico UV-2100 spectrophotometer.

### 2.2. Molecular identification of rhizobia

DNA was extracted from bacterial cultures using SDS/CTAB lysis and phenol/chloroform extraction method (Ausubel et al. 1987). The 16S rRNA gene was PCR-amplified using primer pairs 16S-F10430 = AGAGTTTGATCCTGGCTCAG and 16SR10430 = AAGGAGGTGATTCCAGCC to amplify a nearfull-length, approximately 1500-bp fragment of 16S rDNA from the