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Effect of Combined Inoculation of *Rhizobium* with Soil Yeasts on Nodulation, Growth and Yield of Common Bean (*Phaseolus vulgaris* L.) Under Field Condition

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ABSTRACT

Two field experiments were executed during the seasons of 2012 and 2013 to test response of common bean to inoculation with rhizobia plus soil yeast strains *Saccharomyce cerevisiae*, *Candida sake*, *Saccharomyces exiguous*, *Pichia membranifaciens* and *Cryptococcus lourentii*. The results showed that the improvement of bean nodulation and plant growth parameters by the mixed inoculation treatments with *Rhizobium* plus the yeast strains. The highest improvements recorded for yeast strain *S. cerevisiae* or *S. exiguous*. Inoculation with the yeast strain *S. cerevisiae* mixed with *Rhizobium* induced the following increases in nodule numbers, straw and seed yield: 51.94,10.92 and 16.65% in the firest season and 50.94, 10.32 and 31.37% in the second season, respectively over the inoculation with *Rhizobium* alone. The recorded enhancements are probably due to yeast hormonal production like Indole Acetic Acid (IAA) on root formation growth and lateral roots leading to increased nodulation and nutrient uptake and subsequently increased yield. Also, by increasing mineral nutrients solubilization like phosphorous and iron.

Key words: Common bean, *Rhizobium*, soil yeasts, *Saccharomyce cerevisiae*, nodulation

INTRODUCTION

The utilization of biofertilizers is recently considered a friendly cheap promising alternative of chemical fertilizers application in agriculture, particularly for developing countries. Symbiotic nitrogen fixation is considered the greatest source of satisfying a great part of nitrogen requirement of legume. Bean (*Phaseolus vulgaris* L.) an important leguminous crops, is a subsistence food used as a source of protein (Hemphill and Jackson, 1982). This food legume is known to exhibit a low ability to fix nitrogen (Graham *et al*., 1982; Serraj and Sinclair, 1998). Poor nodulation and variable response to inoculation is mainly attributed to intrinsic characteristics of the host plant, particularly the nodulation promiscuit (Michiels *et al*., 1998) as well as the great sensitivity to other nodulationlimiting factors, such as high rates of N fertilizer used in intensive agriculture, high temperatures and soil dryness (Graham, 1981; Giller and Cadisch, 1995). Genotypic variation in beans as well as compatibility of *Rhizobium*-plant cultivars can also greatly affect the efficiency of symbiosis established.

Increasing attention is new being given to enhance nitrogen fixation by introducing a soil microorganism which is able to stimulate nodulation and nitrogen fixation by *Rhizobium* isolate

use of plant growth promoting rhizobacteria (Figueiredo *et al*., 2008). Yeasts were found in the different soils and rhizosphere of various plants (Rosa *et al*., 1995; Ganter, 2006). Although the numbers of yeasts are low in comparison with other microorganisms, many investigators claimed that this group of organisms seems to play an important role in the soil fertility and they are capable for producing certain growth promoting substances as hormones, amino acid, vitamins, protein, organic acid and soluble and volatile exudates (Mahadevam, 1984; Reed and Nagodawithana, 1991; Sampedro *et al*., 2004; Boby *et al*., 2007). A few studies are available concerning the possibility of increasing the effectiveness of nodulation and N_2-fixation in legumes by soil yeasts. Tuladhar and Rao (1985) reported that combined inoculation of *Rhizobium trifolii* with *Saccharomyces cerevisiae* caused significant increase in the number of root nodules, length of plants and dry weight of Egyptian clover (*T. alexandrinum*) seedlings grown in pots.

The aim of this study was to evaluate the influence of different soil yeasts inocula, *Saccharomyce cerevisiae*, *Candida sake*, *Saccharomyces exiguous*, *Pichia membranifaciens* and *Cryptococcus lourentii* on nitrogen fixation, nodulation, growth and yield of bean (*Phaseolus vulgaris* L.) plants under field conditions.

MATERIALS AND METHODS

Microbial strains used: Five yeast strains (*Saccharomyce cerevisiae*, *Candida sake*, *Saccharomyces exiguous*, *Pichia membranifaciens* and *Cryptococcus* lourenti) were previously isolated from composite sample of the clay soil (Abo-Elyousr and Mohamed, 2009; Hesham and Mohamed, 2011). The strains were maintained on malt-yeast-glucose-peptone agar (YM) slants at 4°C in a refrigerator. *Rhizobium leguminosarum* bv. Phaseoli, locally isolated from root nodules of common bean plants, was used in this study.

Determination of solubilization index: The ability of the yeast strains to solubilize insoluble phosphate was described by the solubilization index: The ratio of the total diameter (colony+halozone) and the colony diameter (Premono *et al*., 1996). Bunt and Rovira (1955) having following composition: 0.4 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 0.1 g $MgCl_2$, 0.1 g $FeCl_3$, 0.1 g $CaCl_2$, 1.0 g peptone, 1.0 g yeast extract, 5.0 g glucose, 250.0 mL soil extracts, 20.0 g agar, 750.0 mL tap water, pH 7.0, was used. Halo zone formation around yeast growth was measured after inoculated on Bount and Rovera agar medium for five days at 25°C.

Determination of soluble P by yeast strains: The ability of the yeast strains to solubilize insoluble tricalcium phosphate was measured in 100 mL aliquots of Pikovskyaya's liquid medium (Rao and Sinha, 1963) having following composition: Glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; KCl, 0.2 g; $MgSO_4.7H_2O$, 0.1 g; $MnSO_4$ trace; $FeSO_4$ trace (pH 7). The yeast strains were grown in 100 mL aliquots of the liquid medium for 5 days at 25°C and then the cultures were filtered and centrifuged at 10000 rpm for 10 min. Soluble phosphorus and pH in the supernatant and blank sample of the medium were determined by the method of Jackson (1973).

Determination of Fe reduction in bulk soil by yeast isolates: With the aim to test the capabilities of isolated yeast isolates to reduce hydroxylamine reducible Fe (III) present in the soil, we used the protocol reported by Lovley and Phillips (1987). Briefly, glass culture tubes containing 1 g of sterile alkaline soil were inoculated with approximately 10^8 CFU g^{-1} soil of *Saccharomyce cerevisiae*, *Candida sake*, *Saccharomyces exiguous*, *Pichia membranifaciens* and *Cryptococcus lourentii* yeast isolates. Uninoculated tubes containing sterile or natural soil were

included as control. The tubes were incubated for 7 days at 30°C in darkness with the soil maintained at field capacity. Fe (II) concentration was determined as reported (Lovley and Phillips, 1987). For this 0.1 g of soil was transferred to 2 mL of HCl 0.5 M in a glass vial, vigorously shaking during 30 sec in a vortex and incubated 1 h at room temperature. An aliquot of 100 mL were mixed with 90 mL of ferrozine solution (0.1% ferrozine, 50 mM HEPES) and the absorbance determined at 562 nm.

Extraction and determination of auxin produced by yeast strains: Extraction of auxins produced in yeast cultures was performed as follows, according to the method described by Strzelczyk and Burdziej (1984). Each of the yeast strains was grown for 5 days at 25°C on YM broth medium. Aliquot of 100 mL of the culture filtrate, was centrifuged at 1000 rpm for 30 min and supernatant was acidified to pH 3 with 1 N HCl then extracted twice with 100 mL of peroxide free ethyl ether in a separatory funnel. The ether extract was then evaporated at 40-45°C to dryness and the residue was dissolved in 2 mL methanol and used for determination of auxins.

Indole Acetic Acid (IAA) was determinated by colorimetric Salkowski reaction (Tang and Bonner, 1947), as follows: Two mL of the prepared methanolic solution (equivalent to 100 mL of the culture) was added to 4 mL of Salkowski reagent (2.025 g $FeCl_3$+300 mL H_2SO_4+500 mL H_2O). The mixture was kept in darkness for 15-30 min before colorimetric reading of the developed rosy color on a Spectronic 20, Baush and Lomb at 530 nm. A standard curve of authentic IAA was performed; consisted of solutions of different concentrations of a pure IAA ranged from 1-10 ppm that were treated with Salkowski reagent as previously described and measured colorimetrically at 530 nm.

Field experiments on bean: During the seasons of 2012 and 2013 a field experiment was conducted at the Experimental Farm of Faculty of Agriculture, Assiut University, to test response of common bean (*Phaseolus vulgaris* L.) to inoculation with rhizobia plus yeasts. The physical and chemical properties of soil are presented in Table 1. The experiment included the following treatments: (1) Seeds inoculated with rhizobia alone, (2) Seeds inoculated with rhizobia plus one of the following yeast strains: *Saccharomyce cerevisiae*, *Candida sake*, *Saccharomyces exiguous*, *Pichia membranifaciens* and *Cryptococcus lourentii*. The area of each plot was 1/400 (10.5 m^2) feddan, containing 6 ridges 60 cm apart. The inoculated seeds were drilled in holes 3-4 cm deep and 15 cm apart on one side of the ridge. Four seeds were placed in each hole (at a rate of 150-160 g $plot^{-1}$), then thinned to two plants after germination. The experimental design was a complete randomized block design with 5 replicates for each treatment.

Table 1: Some physical and chemical characteristics of a representative composite soil sample from the experimental site

Properties	Values
Clay	47.8
Silt	29.5
Sand	22.7
Texture grade	Clayey
Total $CaCO_3$%	2.65
EC dS cm^{-1} (1:1)	1.22
pH (1:1 suspension)	7.47
Total nitrogen (%)	0.06
Organic matter (%)	1.30
Available P mg g^{-1} soil	8.67

Preparation of inoculant and seed treatment: Sterilized peat moss was used as a carrier for inoculant preparations. The pulverized dry peat moss, was neutralized to pH 7 with $CaCO_3$ and $Ca(OH)_2$ and distributed in batches of 50 g each in polyethylene bags and autoclaved for 30 min at 121°C on three successive days. Aliquots of 25 mL of *Rhizobium* or yeast broth culture were used per 50 g of the sterilized carrier material. In case of mixed inoculation with rhizobia and yeast, the single peat inocula were mixed in equal weights just before seed inoculation.

The seeds of each separate plot, in polyethylene bag, were inoculated by adding 10 mL of 40% Arabic gum solution and after mixing, the peat inoculant was added and thoroughly mixed with the seeds until uniformly surface coated. The inoculant was added to seeds at a rate of 15 g/100 g seeds. Peat inocula contained 10^9 viable cells of *Rhizobium* g^{-1} and 10^7 viable cells of yeast g^{-1}.

Plant sampling, growth measurements and yield: Three-plant samples were taken from each plot after 60 days from sowing; the number of nodules, their fresh and dry weights, fresh and dry weights of shoots and roots were recorded. At harvest, the total dry matter yield per plot and seed yield were determined. The nitrogen contents of dried shoots were determined by the semi-micro-Kjeldahl technique (Bremner and Mulvaney, 1982).

Statistical analysis: The data reported in this study were the mean values based on the five replicates. Differences among treatments were tested by ANOVA and mean values among treatments were compared by Duncan's Multiple Range Test at $p = 0.05$. Statistical analysis of the data was performed by using the statistical computer program (Statsoft, 1995).

RESULTS AND DISCUSSION

Solubilization of inorganic phosphates by the yeast isolates: All tested yeast isolates showed varied abilities to solubilize inorganic phosphates as recorded in Table 2, showing different diameter of the clear zone of phosphate desolution on Bount and Rovera agar medium (Fig. 1). The phosphate solubilization index of isolates *Saccharomyce cerevisiae, Candida sake*, *Saccharomyces exiguous*, *Pichia membranifaciens* and *Cryptococcus lourentii* were 1.80, 1.13, 2.09, 2.63 and 2.16, respectively (Table 2). The highest phosphate solubilization index was recorded by the yeast strain *Saccharomyce cerevisiae* and the lowest by the strain *Candida sake*. The strains were further studied for ability to solubilize phosphorus from insoluble phosphate in Pikovskaya's liquid medium containing tricalcium phosphate. Amounts of phosphorus solubilized from tricalcium phosphate by the yeast strains are shown in Table 2. The maximum amount of P solubilized (2.42 mg mL) was recorded for yeast strain *S. cerevisiae* which also recorded the highest phosphate solubilization index, 2.63 (Table 2). The results also show that the lowest amount of P solubilized was recorded by yeast strain *C. sake* (0.65 mg mL^{-1}). The solubilized amounts of P and the

Table 2: Phosphate solubilization and indol acitic acid (IAA) assay by soil yeast strains

Strain	Solubilizing index	Solubilized P in liquid culture (µg mL^{-1})	pH of culture	IAA (µg mL^{-1})
Liquid medium	-	0.009±0.09	6.9±1.430	0.00±0.00
S. cerevisiae	2.63±0.01	2.42±0.030	4.6±0.160	2.57±0.07
C. sake	1.13±0.01	1.54±0.040	5.7±0.120	1.72±0.03
S. exiguous	2.16±0.06	0.65±0.070	6.3±0.810	2.30±0.07
P. membranifaciens	1.80±0.09	2.07±0.050	5.4±0.120	0.87±0.11
C. lourentii	2.09±0.02	2.11±0.030	5.0±0.013	1.01±0.05

All values are the means of three replicates

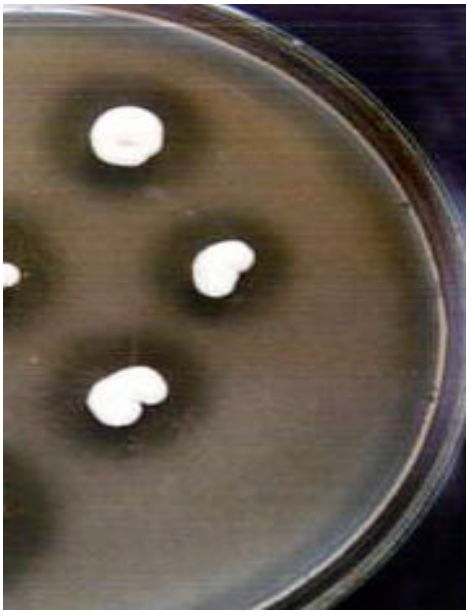

Fig. 1: Colonies of yeast strain *S. cerevisiae* gown for 5 days on Bount and Rovera agar medium showing the clear zone of phosphate dissolution

determined phosphate solubilization index for the yeast strains are compatible with the acidity produced in their cultures (Table 2). This indicates that the organic acids produced from fermentation of sugars in the media by yeast strains are the main cause of solubilization.

Some species of bacteria and other microorganisms are well known to produce organic acids from sugar fermentation and are practically used as biofertilizers for solubilizing inorganic phosphate and increasing phosphorus availability in soils. Other reports have shown the ability of some yeast to solubilize inorganic phosphates. However, great variations in this ability are found between species and strains, as recorded in the present investigation which could be due to variations in kinds of organic acids or amounts produced. Kanti and Sudiana (2002) found that nine yeast strains out of the twenty three isolates, belonged to genera of *Debaromyces, Pichia, Rhodotorula* and *Candida* that were isolated from soil, had the ability to dissolve $Ca_3(PO_4)_2$. Vassileva *et al.* (2000) pointed to the importance of yeasts, as well-known, is the production of organic acids (especially citric) and their high survival rate under extreme soil conditions, in transformation of rock phosphates and insoluble carbonate leading to increases in available phosphorus, Fe and other micronutrients. They recorded the simultaneous solubilization of rock phosphate and calcium carbonate by free and agar encapsulated cells of yeast strain *Yarowia lipolytica* as a result of citric acid production in repeated batch-shake-flask fermentation medium. They indicated that *Yarowia lipolytica* and other acid producing yeasts could be successfully applied for rock phosphate solubilization and in preparation of soil inoculants.

Fe reduction by yeast isolates in alkaline soil: The Fe-reducing abilities of yeast isolates were tested in tubes containing sterilized natural soil. A group of tubes were inoculated with the different yeast isolates or with natural alkaline soil with its native microbial population and uninoculated tubes containing sterilized alkaline soil were inoculated as control. The Fe (II) content in the tubes

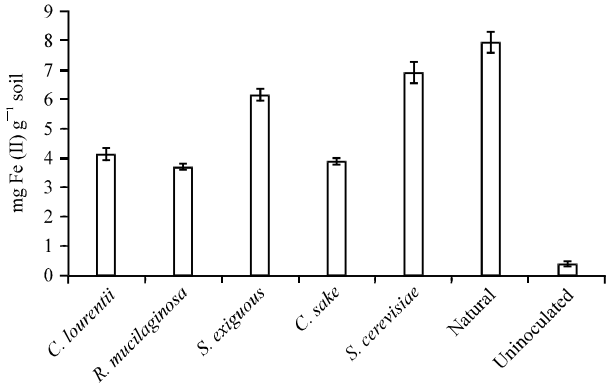

Fig. 2: Iron reduction ability of soil yeast strains in alkaline soil

was further measured 7 days after inoculation by the ferrozine assay (Lovley and Phillips, 1987). As shown in Fig. 2 Fe-reducing by the yeast isolates were 6.9, 3.9, 6.13, 3.70 and 4.13 mg kg^{-1} soil for the yeast strains *Saccharomyce cerevisiae, Candida sake, Saccharomyces exiguous, Pichia membranifaciens* and *Cryptococcus lourentii,* as compared with 7.93 and 0.40 mg kg^{-1}, respectively reduced by natural (nonsterilized alkaline soil) and uninoculated (sterilized alkaline soil). The strain *S. cerevisiae* and *S. exiguous* showed the greatest Fe(II)-reducing activity among the different yeast strains when compared to the control.

Microorganisms are crucial partners of plant to access Fe in the rhizoshere, they can improve plant growth through a continuous and efficient Fe supply for growth and chlorophyll synthesis. Different mechanisms have been suggested by which microbial species enhance Fe availability to plant. For instance, some species produce and secrete siderophores that mobilize Fe from soil to roots (Masalha *et al*., 2000; Rroco *et al*., 2003). Some microbial species forming 2-ketogluconate or poly-basic organic acids as tartaric and citric, oxalic, formic and glycolic acids are found more effective in chelating Ca and Fe than other organic acids (Banik and Dey, 1982; Stevenson, 1982). In addition, certain bacterial species are able to transfer electrons from their metabolism to Fe(III) oxides in a dissimilatory Fe-reducing process (Lovley, 1991).

Production of auxin (IAA) by the yeast isolates: The ability of the yeast strains grown on YM liquid medium to produce Indol Acetic Acid (IAA) was tested. Data in Table 2 show the amount of Indol Acetic Acid (IAA) produced by yeast strains on YM medium after 7 days of incubation at 25°C. The results show a great variation among the tested yeast strains in production capacities of this growth promoting substance, from 0.87 to 2.57 μg mL^{-1}. The results also show that the strain *Saccharomyce cerevisiae* produced the highest amounts of IAA while the lowest amounts produced by the strain *Candida sake*.

Some reports have shown that yeast is one of the microorganisms which are able to produce growth promoting substances as hormones, amino acid and vitamins (Mahadevam, 1984; Reed and Nagodawithana, 1991; Nassar *et al*., 2005).

Response of bean to inoculation with *Rhizobium* plus yeast strains under field condition: The field experiments were conducted at the Experiment Farm of the Faculty of Agriculture, Assiut University during the season of 2012 and 2013 to study the response of bean (*Phaseolus vulgaris* L.) cv. Giza-6 to mixed inoculation with *Rhizobium* plus the soil yeast strains.

Table 3: Effect of inoculation with *Rhizobium* and soil yeast strains on nodulation, growth and yield of common bean (season, 2012)

			Dry weight (g plant^{-1})			Yield (kg fed^{-1})	
Inoculation treatment	Nodules (No. plant^{-1})	Nodules dry weight (mg plant^{-1})	Shoot	Root	Shoot N (mg plant^{-1})	Straw	Seed
R+*Leguminosorum* (R)	16.96^{d}	60.16^{b}	3.85^{c}	1.50^{bc}	111.98^{d}	1180.1^{e}	607.6^{d}
R+*S. cerevisiae*	25.77^{a}	71.20^{a}	5.84^{a}	2.36^{a}	130.61^{a}	1309.0^{a}	708.8^{a}
R+*C. sake*	21.18^{b}	62.93^{b}	4.22^{bc}	1.93^{ab}	121.77^{b}	1192.8^{d}	667.6^{b}
R+*S. exiguous*	19.11^{c}	60.50^{b}	4.30^{bc}	1.40^{c}	115.37^{c}	1231.3^{b}	661.5^{b}
R+*P. membranifaciens*	18.24^{cd}	61.03^{b}	4.02^{bc}	1.94^{ab}	119.97^{b}	1185.6^{de}	613.0^{d}
R+*C. lourentii*	17.58^{cd}	60.65^{b}	4.69^{b}	1.79^{bc}	115.15^{c}	1213.2^{c}	651.3^{c}
CV (%)	10.48	8.88	13.11	9.75	16.22	18.340	20.30

Values presented are treatment means 5 replicates. In each column the means followed by the same letters do not differ statistically (p = 0.05) from each other, according to Duncan's multiple range test

Table 4: Effect of inoculation with *Rhizobium* and soil yeast strains on nodulation, growth and yield of common bean (season, 2013)

			Dry weight (g plant^{-1})			Yield (kg fed^{-1})	
Inoculation treatment	Nodules (No. plant^{-1})	Nodules dry weight (mg plant^{-1})	Shoot	Root	Shoot N (mg plant^{-1})	Straw	Seed
R+*Leguminosorum* (R)	22.26^{d}	59.47^{d}	4.10^{b}	1.65^{c}	113.42^{e}	1184.2^{e}	613.2^{e}
R+*S. cerevisiae*	33.60^{a}	69.35^{a}	5.45^{a}	2.15^{a}	134.42^{a}	1306.5^{a}	805.6^{a}
R+*C. sake*	25.35^{b}	61.62^{c}	4.75^{b}	1.98^{b}	120.40^{b}	1193.9^{d}	669.7^{bc}
R+*S. exiguous*	24.44^{cd}	62.90^{bc}	4.76^{b}	1.84^{c}	116.80^{d}	1227.4^{b}	692.0^{c}
R+*P. membranifaciens*	24.20^{bc}	61.30^{bc}	4.50^{b}	1.95^{b}	119.12^{c}	1205.5^{c}	678.9^{b}
R+*C. lourentii*	23.85^{d}	59.67^{b}	4.42^{b}	1.73^{c}	115.47^{d}	1210.0^{b}	656.5^{d}
CV (%)	8.09	11.37	10.46	7.59	13.59	15.4	18.3

Values presented are treatment means 5 replicates. In each column the means followed by the same letters do not differ statistically (p = 0.05) from each other, according to Duncan's multiple range test

The data obtained in the season 2012 (Table 3) show the improvement of bean nodulation and plant growth parameters by the mixed inoculation treatments with *Rhizobium* plus the yeast strains. The highest improvements recorded for yeast strain *S. cerevisiae* or *S. exiguous*. Inoculation with the yeast strain *S. cerevisiae* mixed with *Rhizobium* induced the following increases in shoot dry weight, root dry weight, nodule numbers, nodule dry weigh and N-uptake in shoot: 51.68, 57.33, 51.94, 18.35 and 16.63%, respectively and resulted in 1092 and 1665% increase in straw and seed yield over the single inoculation with *Rhizobium* alone.

In the second season (Table 4), exactly the same trends were recorded for effects of yeast strains inoculation with rhizobium on growth parameters in addition to inducing highly significant increases in seed yield with yeast strain *S. cerevisiae* and *S. exiguous*. Also strain *S. cerevisiae* was the most stimulate, in the second season, the % increases, in shoot dry weight, root dry weight, nodule numbers, nodule dry weigh, N-uptake in shoot, straw and seed yield induced by yeast strain *S. cerevisiae* mixed with rhizobium, respectively were 51.94, 30.31, 20.49, 16.61, 18.51, 10.32 and 31.37% compared with the single inoculation with *Rhizobium*.

The recorded enhancements are probably due to yeast hormonal effects on root growth and lateral roots leading to increased nodulation and nutrient uptake and subsequently increased yield. Also, by increasing mineral nutrients solubilization like phosphorous and iron. The variation in amount of improvements induced by the different yeast strains, point to the importance of selecting the most compatible efficient strain for inoculation with the *Rhizobium* species. In case of bean, the most stimulative yeast strain compatible with inoculated *Rhizobium leguminosarum* bv. Phaseoli was *S. cerevisiae*.

It could be conducted from the results obtained in the experiment indicate that the presence of yeast with the specific *Rhizobium* strain in the inocula could significantly influence the specific *Rhizobium*-legume symbiotic interaction leading to enhanced nodulation and improvement of plant growth and yield. Similar results were reported by Tuladhar and Rao (1985), who reported that seed inoculation of five legumes (*Vigna mumgo, Vigna aureus, Vigna unguiculata, Glycine max* and *Leuceana leucocephala*) with *Saccharomyces cerevisiae* cells alone significantly enhanced nodulation of all five legumes by native rhizobia and significantly increased plant growth and grain yields. Also, the bio-organic treatment consisted of 10 or 5 m^3 tarmyard manure FYM+*Rhizobium leguminosarum*+soil yeast *Candida tropicalis* increased plant height, leaves numbers, leaves fresh weight, pods number $plant^{-1}$, seed number pod^{-1}, pod weight and protein content of faba bean plants compared with the positive control (Mohamed and Gomaa, 2005).

Ali *et al.* (2008) reported that the application of auxins (IAA or 4-CL-IAA) improved the growth, leghemoglobin content, the number of nodules and nitrogenase activity in mung bean plants which would ultimately leads to better seed yield.

CONCLUSION

Co-inoculation of common bean with *Rhizobium leguminosarum* and soil yeast strain (*Saccharomyces cerevisiae*) resulted in higher nodule numbers, nodule dry weigh, N-uptake, straw and seed yield over the single inoculation with *Rhizobium* alone. The recorded enhancements are probably due to yeast hormonal effects on root growth and lateral roots leading to increased nodulation and nutrient uptake and subsequently increased yield. Also, by increasing mineral nutrients solubilization like phosphorous and iron. The variation in amount of improvements induced by the different yeast strains, point to the importance of selecting the most compatible efficient strain for inoculation with the *Rhizobium* species.

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