

Influence of Minerals Fertilizer Addition and Inoculation Methods on Nodulation and Growth of Two Introduced *Acacia* species

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The influence of different mineral fertilizers ((P: K 1:1 and NPK 1:1:1) on nodulation and growth of two introduced *Acacia* species grown under nursery conditions was investigated. Two *Acacia* species; *Acacia ampliceps* and *A. salicina* were treated with combinations of N, P, and K fertilizers and two inoculation methods (seed inoculation and soil inoculation). Nodulation status (nodule number and nodules dry weight) in roots and plant growth parameters (seedling length, collar diameter, and shoot and root dry weight) were recorded 120 days after inoculation. Soil inoculation with *Rhizobium* in combination with mineral fertilizer significantly affected growth and nodulation compared with soil inoculations alone. Seeds inoculated with PK+ *Rhizobium* performed better than other treated seeds and resulted in seedlings with the most growth and root nodules. The *Rhizobium* strain LLR14 was highly effective in both *Acacia* seedlings. *Acacia ampliceps* was more responsive than *A. salicina* to *Rhizobium* inoculation in combination with mineral fertilizer treatments. The use of fertilizers and inoculation in the nursery may facilitate the production of superior seedlings with high growth potential. *A. ampliceps* and *A. salicina* seedlings inoculated with *Rhizobium* and treated with P or K fertilizer may facilitate afforestation and reforestation programs and reducing the time needed to achieve canopy closure, particularly in the arid regions.

Keywords: Introduced *Acacia* species, Inorganic fertilizer, Inoculation methods, Nursery conditions.

Chemical fertilizers have been used for plant cultivation because of their high solubility and positive impacts on crop yield. Inorganic fertilizers may influence plant adaptation and their ability to dominate and flourish in legume production^{1, 2}. Mineral fertilizers are also an important factor in modern agriculture;

approximately 60% of humanity currently owes its nutritional survival to nitrogen (N) fertilizers. Growing concern about the environmental consequences of mineral N use emphasize the need to develop new production technologies that are sustainable for economically and ecologically³. While application of N fertilizers to seedlings can increase N₂ fixation through a starter effect, such a method is costly; with high ATP needs, N₂ fixation generally has a high requirement for the mineral phosphorus (P) and P adjustments on low-P soil increases biological N fixation rates^{4, 5}. Conversely, N₂-fixing tree species may compete with non-fixing

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species for P in afforestation, ultimately reducing soil P availability⁶ and necessitating requiring additional P adjustments. Although the addition of P to P-poor soils usually lead to increased N₂ fixation, this has sometimes also encouraged tree growth; in dryland West Africa, for example, as little as 30 kg P ha⁻¹ enhanced the growth of woody legumes⁴.

Acacia ampliceps (Maslin.) and *Acacia salicina* (Lindley) belong to the legume family. They are thornless species native to Australia that were introduced to Saudi Arabia in the 1990s. Both species grow as fast-growing dense shrubs or small trees (5–20 m tall) with a spreading crown. They are able to grow in the arid and semi-arid regions of Saudi Arabia and considered to be one of the most drought- and salt-tolerant species that are able to grow on a wide range of soils. Furthermore, these *Acacia* species are highly adapted to low rainfall and high temperature conditions, allowing them to grow in degraded soils with poor fertility levels⁷. These two *Acacia* species are found on sand plains, flood plains, and along drainage lines and are important sources of fodder, fuel, and timber^{8,9,10}. They can form a symbiotic association with rhizobia that encourages the formation of nodules where atmospheric N is fixed¹¹. These two species were selected for their prominence in the afforestation and reforestation of the central region of Saudi Arabia.

The importance of woody-legume–*Rhizobium* symbioses, which are able to fix considerable quantities of N₂ and serve as a cheap fertilizer replacement, has been frequently reported for land reclamation and land improvement, particularly in arid regions with high salinity, low fertility, and drought periods. The majority of leguminous plant form symbiotic relationships with members of genera that belong to the class Alphaproteobacteria (*Allorhizobium*, *Azorhizobium*, *Blastobacter*, *Bradyrhizobium*, *Devosia*, *Ensifer*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium*, and *Sinorhizobium*). Some legumes, such as those belonging to the *Mimosa* genus, are nodulated mostly by members of the class Betaproteobacteria in the genera *Burkholderia* and *Cupriavidus*. Recently, the endemic Mexican mimosas were shown to be nodulated predominantly by *Alphaproteobacteria* from the genera *Rhizobium*

and *Ensifer*^{12,13}. Work on *Rhizobium*-legume symbiosis has been conducted over many years; several studies examining rhizobia isolated from tree legumes have indicated that there is considerable phenotypic and genotypic diversity among strains, although less attention has been given to interaction symbionts of introduced *Acacia* species¹⁴. *Acacia* species can assist understory plants by N fixation^{15,16}. The growth of understory plants will develop the plant community by modifying physical and chemical properties of the soil beneath the canopy¹⁷.

Inoculation of legume seeds is an efficient way to introduce effective *Rhizobium* to the soil and to the rhizosphere of legumes¹⁸. The efficacy of inoculation varies depending the number of viable *Rhizobium* available to infect the legume roots. Previous studies in nursery conditions have indicated that it was possible to improve the growth of *Acacia* species by inoculating them with effective micro symbionts¹⁹. Inoculation does result in higher numbers of viable rhizobia on seeds and there is substantial demand for commercially inoculated legume seeds. There are a number of different methods that can be used depending on the seed size and availability of equipment. Sarr et al.²⁰ demonstrated that the improvement in growth of *Acacia* species was more marked if the inoculation of trees was carried out using dissolved alginate beads containing a mixture of selected rhizobial strains; there is, however, no information regarding such growth responses in natural environments. Many factors in the field are related to the success of the rhizobia inoculation, including drought, soil fertility, the genetic source of the host plant, and the ability of rhizobia present in the soil to infect the host plant in the presence of host-compatible native rhizobial strains²¹. Native strains of rhizobia can negatively affect inoculation and the majority of isolates forming nodules on field-grown plants are usually relatively poor N fixers²². The competitiveness of a rhizobial strain contained in an inoculum in terms of host-plant nodulation must be assessed before unsterilized soil containing a large population of native rhizobia compatible with the target host plant is inoculated. Under greenhouse conditions, Lesueur and Diouf²³ showed significant growth response differences between two variants of *Calliandra calothyrsus* inoculated with a rhizobial strain. Likewise, Sarr et

al.²⁰ demonstrated a strong interaction effect on tree growth between *A. senegal* and *A. nilotica* with a mixture of rhizobial strains.

The objective of the current study was to establish guidelines to examine and optimize root nodule formation in two introduced *Acacia* trees by investigating the inoculation methods of *Rhizobium* in conjunction with subsequent mineral fertilizer treatments, ensuring high growth rates in individual trees under Riyadh region conditions.

MATERIALS AND METHODS

Experimental procedures

This study was carried out at the nursery of the Range and Forestry Applied Research Unit at the Experimental Station of the Food and Agricultural College at Dirab during the two growth seasons of 2015 and 2016. Seeds of *A. ampliceps* (Maslin.) and *A. salicina* (Lindley) used in this study were obtained from the Range and Forestry Applied Research Unit, and Dirab Valley, South of Riyadh City. The characteristics of the soil used in the experiments are shown in Table (1).

Mineral fertilizers and inoculation methods

Three common mineral fertilizers, ammonium nitrate (33% N) as a source of N, 0.3 g/plant; superphosphate (15% P₂O₅) as a source of P, 0.33 g/plant; and potassium sulfate (50% K₂O) as a source of K, 0.1 g/plant, in addition to two different mix fertilizer treatments; PK 1:1 and NPK 1:1:1. were used in the study. For each fertilizer treatment, the dose was divided into two parts, the first dose was administered in June and the second was in July in each growing season. Two inoculation methods a; seed inoculation by adding a 250 ml of bacteria mixture with sugar solution, and activated charcoal, conducted during germination in March, b; soil inoculation, where the soil of each plant was injected with 15 ml of rhizobium after seed germination

Rhizobium strain used

Seedlings of *A. ampliceps* (Maslin.) and *A. salicina* (Lindley) were inoculated with strain *Rhizobium* strain (LLR14) isolated from the roots of *Leucaena leucocephala* trees grown in forestry nurseries at the Experimental Station of the Food and Agricultural College at Dirab. Bacteria were isolated from surface-sterilized nodules using the standard procedure of Vincent,²⁴ and cultured in

yeast extract–mannitol agar (YMA) medium. All the isolates were subcultured and then incubated on YMA medium at a 30°C and kept at 4°C; isolates were stored long-term in 20% glycerol at “80°C. The strain used in the study (Table 2) was a fast-growing type belonging to the *Sinorhizobium fredii* strain NGR 234 (data not published).

Seedling production and infection assay

Seeds of *A. ampliceps* and *A. salicina* were soaked in hot water (100°C) for 15 min and cool water (20°C) for 24 h, to prevent the inhibitory effect of seed coats on germination. Seeds of *A. ampliceps* and *A. salicina* then sown in sterilized medium (autoclaved at 121°C for 1 h) containing a mixture of sand and vermiculite (2:1 v/v) in the greenhouse, maintained at day/night temperatures of approximately 25°C/17°C. After germination, the seedlings were transplanted into pots (15 cm diameter) containing 2 kg of mixed sterilized sand and vermiculite. Pots were organized in a split-split plot in a randomized complete block design in a greenhouse. The *Acacia* seedlings were inoculated with 15 ml of mature *Rhizobium* isolates (LLR14; approximately 10¹⁰ bacterial cells ml/plant) grown in yeast extract mannitol broth culture incubated at 28°C with shaking (200 rpm) for 5 days. Two methods of infection were used to inoculate the seedlings, seed inoculation and soil inoculation.

The seedlings were placed in a greenhouse between March 2015 and the first week of May 2016. The daily maximum temperature ranged from 25 to 30°C and the minimum temperature from 19 to 20°C. The treatments used in the study included; a) control treatment; b) inoculation with *Rhizobium* only using seed inoculation and/or soil inoculation; c) inoculation with *Rhizobium* using seed inoculation and/or soil inoculation with treatment with PK 1:1 fertilizer; and d) inoculation with *Rhizobium* using seed inoculation and/or soil inoculation with treatment with NPK 1:1:1 fertilizer. Seedlings were maintained in the greenhouse during the experiment and were watered every other day with tap water. Seedlings were harvested 120 days after inoculation, and the following measurements were recorded: seedling height and diameter shoot and root dry matter per seedling, number of nodules per seedling, and nodule dry weight per seedling. Seedlings were dried at 70°C and the macro and micronutrients were measured. Total N content was determined

using the Kjeldahl method. K⁺ was determined using flame photometry (Corning 400, Sherwood Scientific Ltd, Cambridge, UK), and P was measured using colorimetric determination.

The experimental design

The split-split plot system in a randomized complete block design was used in this experimental according to Steel and Torrie²⁵; the main plot was the tree species, the subplot was fertilizer treatments, and the sub-subplot was *Rhizobium* isolates. Three replicates were used for each treatment. Statistical analysis was done using ANOVA, F-tests, and Least significant differences

available within the SAS software package program²⁶ (version 9.13 2008). The combined analysis was used for the two growing seasons.

RESULTS AND DISCUSSION

Growth parameters

The average seedlings height of *A. ampliceps* was taller than *A. salicina* (Table 3). *A. ampliceps* seedlings were taller on average than *A. salicina* seedlings (Table 3). The most effective treatment was PK fertilizer in combination with *Rhizobium* (with average 19.83 cm) followed by

Table 1. Physical and chemical characteristics of the soil used in the study

Particle size distribution (%)			Soil texture	pH	EC [†] (ds m ⁻¹)	Soluble cations (mg L ⁻¹)			Soluble anions (mg L ⁻¹)		Available nutrients (mg kg ⁻¹)		Organic Mater OM %
sand	silt	clay				Na ⁺	K ⁺	Ca ²⁺	SO ₄ ⁻²	CL ⁻	N	P	
78.7	14.0	7.3	Sandy loam	8.65	1.45	1.04	0.15	6.10	6.0	2.2	13.0	0.15	1.0

[†]EC: electrical conductivity

Table 2. Characterization of nodule-forming isolates from *Leucaena leucocephala*

Host plant	Strain	Source of Strain	Growth period (hours)	Generation time (hours)	<i>Rhizobium</i>	Identity Query (bp)	Locus
<i>Leucaena leucocephala</i>	LLR14	Wadi Dirab	3–5	2.15	<i>Sinorhizobium fredii</i>	899	CP001389

Table 3. Mean growth parameter values of tree species treated with fertilizers and *Rhizobium* over two growing seasons

Tree species	Treatments	Growth parameters				Nodulation	
		Height (cm)	Diameter (cm)	Shoot dry weight (g)	Root dry weight (g)	Number of nodules	Nodules dry weight (g)
<i>Acacia ampliceps</i>	Control	6.64b	0.08b	3.30d	1.29d	0.0d	0.0b
	<i>Rhizobium</i>	9.15b	0.08b	12.54c	10.45c	37.2b	0.3a
	PK+ <i>Rhizobium</i>	19.83a	0.17a	35.49b	17.17a	46.9a	0.4a
	NPK+ <i>Rhizobium</i>	17.37a	0.12b	43.10a	12.88b	10.3c	0.1a
Mean		13.45	0.11	23.61	10.45	23.61	0.2
<i>Acacia salicina</i>	Control	5.86b	0.04b	2.21b	0.8c	0.0d	0.0b
	<i>Rhizobium</i>	6.17b	0.06a	3.79b	1.95b	22.1b	0.12a
	PK+ <i>Rhizobium</i>	13.92a	0.09a	21.82a	14.11a	31.4a	0.25a
	NPK+ <i>Rhizobium</i>	12.43a	0.10a	21.60a	13.23a	8.6c	0.06a
Mean		9.74	0.72	12.36	7.51	15.25	0.11

NPK with *Rhizobium* whereas the control resulted in shorter seedlings with an average height of 6.64 cm (Table 3). On the other hand, no significant

differences ($p < 0.05$) in growth height were found between the two inoculation methods (Table 4).

The analysis of variance of the stem diameter showed highly significant differences (p

Table 4. Mean growth parameter values of tree species inoculated with *Rhizobium* using two different methods in combination with the fertilizer treatments across the two growing seasons

Tree species	Inoculation method	Growth parameters				Nodulation	
		Height (cm)	Diameter (cm)	Shoot dry weight (g)	Root dry weight (g)	Number of nodules	Nodules dry weight (g)
<i>Acacia ampliceps</i>	Seed inoculation	7.95a	0.08a	12.79a	8.0a	18.58a	0.21a
	Soil inoculation	7.33a	0.08a	13.98a	6.79a	6.75b	0.06b
Mean		7.64	0.08	13.39	7.40	12.67	0.14
<i>Acacia salicina</i>	Seed inoculation	9.55a	0.05a	9.40a	4.95a	15.0a	0.05a
	Soil inoculation	8.16a	0.06a	8.0a	5.04a	8.1b	0.05a
Mean		8.86	0.06	8.7	5.0	11.6	0.05

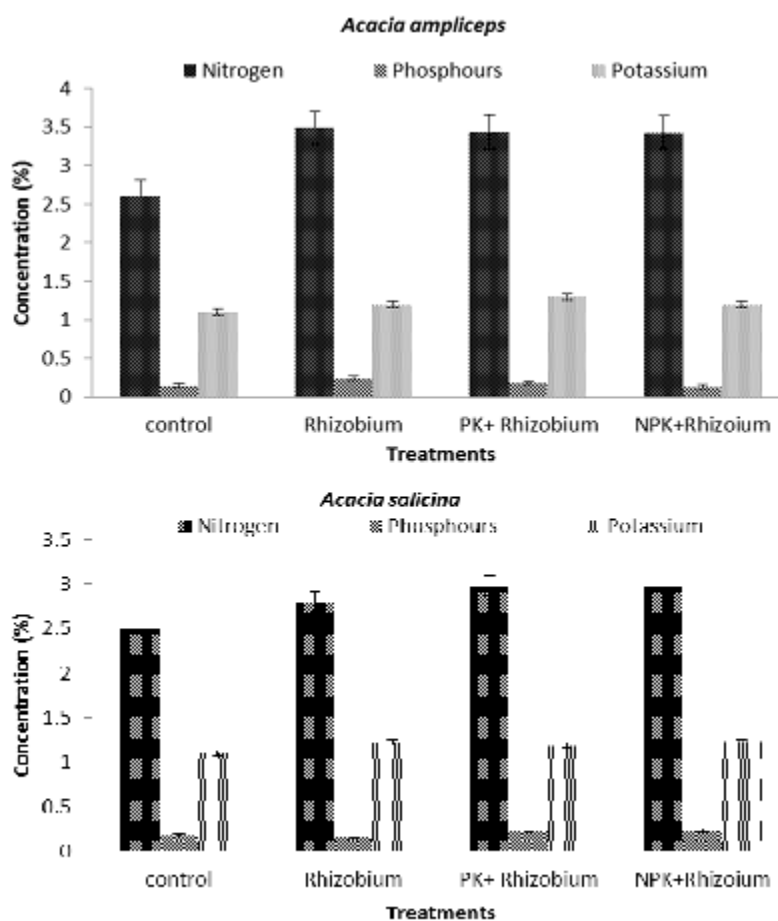


Fig. 1. The effect of fertilizer treatments on the macronutrients concentration (%) of *Acacia* species in the two growing seasons

<0.05) between tree species and fertilizer treatment, while no significant differences were found between inoculation methods. The average stem diameter indicated that *A. ampliceps* seedlings had smaller stem diameters than *A. salicina* seedlings. The fertilizer treatments showed that PK with *Rhizobium* resulted in the widest stem diameter followed by NPK with *Rhizobium* (Table 3). The stem diameter results indicated that the PK and NPK treatments had different effects on the stem diameter; PK treatment with *Rhizobium* resulted in a larger average of stem diameter in *A. ampliceps*, while NPK treatment resulted in a larger stem diameter in *A. salicina* in both growing seasons. The shoot and root dry weight varied between the two *Acacia* species whereas the inoculation method did not significantly affect the measured growth parameters.

For the shoot and root dry weights, highly significant differences ($p < 0.05$) were found between tree species, fertilizer treatments, and their interactions over both growing seasons. *A. ampliceps* seedlings had higher shoot and root dry weights with either PK or NPK fertilizer in combination with *Rhizobium* than *A. salicina* (Table 3). The shoot and root dry weight varied between the two inoculation methods in the *Acacia* species. Soil inoculation resulted in higher shoot dry weight in *A. ampliceps*, whereas shoot dry weight of *A. salicina* was higher with the seed inoculation method. Conversely, the root dry weight was higher with seed inoculation in *A. ampliceps* and with soil inoculation in *A. salicina* (Table 4).

The results from the two seasons indicated that *A. ampliceps* was more responsive

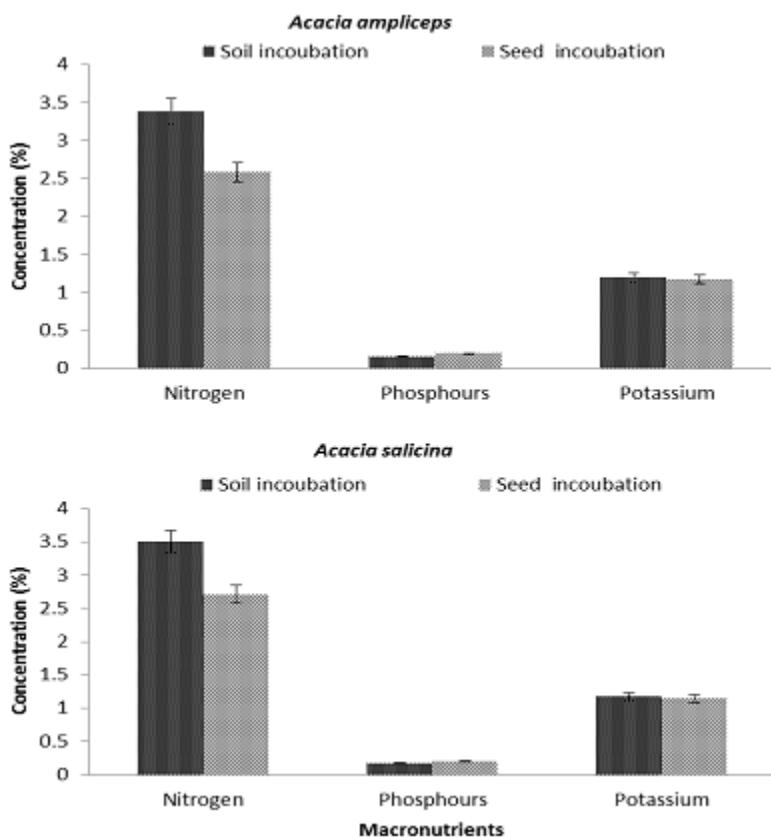


Fig. 2. The effect of inoculation method on the macronutrients concentration (%) of *Acacia* species in the two growing seasons

to fertilizer treatment and *Rhizobium* inoculation method than *A. salicina*. Treatment with PK fertilizer and *Rhizobium* was the most effective compared with the compared with NPK and control used in this study. herefore, the findings of this study are consistent with the results obtained by Ahmadi and Chaichi²⁷, Bekere and Hailemariam²⁸, and Huda et al.⁵, who showed that, the inoculation in combination with chemical fertilizer application resulted in greater concentrations of the nutrients and increased plant growth. The application of fertilizer significantly affected every measured parameter; fertilized seedlings were significantly taller^{29,30}. Inoculation with *Rhizobium* in addition to the application of fertilizer further improved the measured growth parameters. This result was consistent with the results obtained by Shetta³¹, who found that the native *Rhizobium* increased the height growth of *A. karroo*, but contradicted with the results obtained by Sanchez et al.³², who indicated that native *Rhizobium* populations were not effective enough to obtain a significant increase in shoot height and nodulation. On Contrary Ahmad et al.³³, found that seed inoculation with *Rhizobium* significantly increased plant height in many legumes. The fertilizer rate significantly affected root diameters of *Acacia koa*²⁹. Moreover, the stem diameter increased following inoculation with *Rhizobium* strains using the soil inoculation method compared to the seed inoculation method. The inoculation efficiency varied according to several factors that affected the number of viable rhizobia available for root legume infection¹⁸. In this study, inoculation with *Rhizobium* was found to increase the root diameter. This was consistent with the findings of Deaker et al.¹⁸ and Molla et al.³⁴, who indicated that the root growth and nodulation were significantly increased, but contradicted with the results obtained by Philpotts³⁵. The seed inoculated plants exhibited significantly greater root and shoot mass as compared with the control plants. Seed inoculation has, however, been shown to result in decreasing bacterial rates^{36,33}. The enhancement of growth parameters observed following inoculation. In this study the results were consistent with the results obtained by Shaheen and Rahmatullah³⁷, Bekere and Hailemarion²⁸, and Ali et al.³⁸ who showed that seed and soil inoculations combined with fertilizer

treatments increased roots and shoots dry matter of legumes.

The nodulation of *Acacia* species

The results obtained in the present study indicated that both the *Acacia* species were able to form nodules with *Rhizobium* strains under all fertilizer treatments except the control treatment. The nodule number and nodule dry weight analyses revealed that highly significant differences ($p < 0.05$) were found between tree species, fertilizer treatments, inoculation methods and their interactions. *A. ampliceps* was more responsive to fertilizer treatments and *Rhizobium* inoculation than *A. salicina*. Furthermore, the PK fertilizer treatment in conjunction with *Rhizobium* inoculation resulted in a higher number of nodules when compared with other fertilizer treatments in both *Acacia* species (Table 3). The effect of inoculation method on nodule number indicated that the seed inoculation method was more effective than the soil inoculation method in both *A. ampliceps* and *A. salicina* over the two growing seasons (Table 4). Consistently, the nodule dry weight of *A. ampliceps* was higher than *A. salicina* in both growing seasons. Similarly, the PK fertilizer treatment in conjunction with *Rhizobium* inoculation resulted in higher nodule dry weight compared with NPK in both *Acacia* species (Table 3). The inoculation method showed that seed inoculation was more effective in terms of increasing the nodule dry weight than the soil inoculation method in *A. ampliceps*. In *A. salicina* no differences were observed between the two inoculation methods (Table 4).

The seed inoculation method had a greater effect on root nodules and the dry weight of nodules than soil inoculation. The results obtained from our results are consistent with the results of previous findings of Huda et al.⁵ and Bekere and Hailemariam²⁸ who indicated that fertilizer with P and K without N increased the nodule number and nodule dry weight as well as the total N in shoots. The applied N fertilizer significantly and linearly reduced the nodule number and fresh and dry weight of nodules. Achakzai³⁹ found that legume group fertilized with NPK had decreased nodulation and a reduced number of nodules. Vessey⁴⁰ demonstrated that the application of N fertilizer to inoculated legumes

was unnecessary when good quality inoculant was used. The inoculation method did not influence any of the growth parameters or nodule formation; this because of the effective indigenous rhizo-competitions between microorganisms in the soil and *Rhizobium*²⁹. The fertilizer treatments affected the number of nodules, and the nodule dry weight. This finding was consistent with the findings of Ngwu², who found that the legumes ability to nodulate was affected by NPK treatments at various levels. He also indicated that fertilizer application at various rates affected nodulation and plant height. Ali et al.³⁸ indicated that P application along with *Rhizobium* inoculation increased significantly the dry weight of nodules. Similar results were obtained by Javaid¹.

Nodule number was significantly enhanced by inoculation with *Rhizobium* in combination with PK fertilizer. These results differ from those obtained by Umamaheswari et al.⁴¹ who found that the number of nodules was not significantly influenced by different fertilizer treatments. The current study indicated that seed inoculation was the most effective method to infect the roots of *A. ampliceps* and *A. salicina* seedlings; this result is in accordance with those obtained by Ahmed et al.³⁶ and Huda et al.⁵ who found that the inoculation of mung bean and *Dalbergia sissoo* seeds with *Rhizobium* significantly increased nodulation and plant height. Researches also reported that seed inoculation and N fertilizer significantly increased the number of nodules, the fresh and dry weight of nodules, and the biological yield. Seed, soil, and seed + soil inoculations increased the plant parameters and shoot dry matter²⁸.

Macronutrients accumulation in *Acacia* species

In both seasons, the N, P, and K concentrations (%) in *Acacia* seedlings varied significantly ($p < 0.05$) among tree species, fertilizer treatments, and inoculation methods. The average concentration in *A. ampliceps* seedlings was higher for N, P, and K (%) compared with *A. salicina* (Figure 1). In *A. ampliceps* seedlings the *Rhizobium* treatment gave the highest of N, P concentrations (%) while the PK + *Rhizobium* had the highest K concentration (%) compared with the control treatment. The NPK + *Rhizobium* treatment in *A. salicina* had highest concentrations of N, P and K (%) compared with the other treatments (Figure 1).

For the inoculation method, the obtained results showed that soil inoculation was associated with higher N, P and K concentrations (%) than seed inoculation across the two growing seasons (Figure 2). *A. ampliceps* was more responsive to the soil inoculation and had a higher N and K concentration (%) than *A. salicina*. The P concentration (%) differed between the two inoculation methods in the *Acacia* species (Figure 2). Generally, the data from the two seasons showed that N, P and K concentrations varied among the tree species with different fertilizer treatments and inoculation methods. Fertilizer has previously been shown to increase foliage N content, particularly in *Elaeagnus angustifolia*⁴,⁴². Villar-Salvador et al.³⁰ found that seedlings given high rates of fertilizer in the nursery had greater N concentrations than seedlings given low rates of fertilizer either with or without rhizobial inoculation. Also, Oliet et al.⁴³ recommended the application of organic and/or inorganic fertilizers, including P, to nursery-grown saplings of *A. salicina* to ease the stress of seedling growth. Particularly in nutrient-poor soils in semi or arid environments, such additions increased long-term plantation establishment. These results demonstrated an actual interaction between inoculated rhizobial methods, and host plants in terms of growth, competitiveness, and nodulation.

CONCLUSIONS

The results of this study concluded that inoculation of soil with *Rhizobium* in combination with fertilizer treatment significantly affected growth and nodulation of *Acacia* species compared with soil inoculation alone. Seed inoculation with PK + *Rhizobium* performed better than other treatments and produced the greatest amount of growth and root nodulation. The *Rhizobium* strain was highly effective on both introduced *Acacia* seedlings studied here. *A. ampliceps* was more responsive to inoculation with *Rhizobium* in conjunction with the fertilizer treatments compared with *A. salicina*. Concurrent use of fertilizers and inoculation in the nursery, may assist in the production of superior seedlings that may reduce the time needed to achieve canopy closure, thereby helping achieve restoration objectives more rapidly. The study suggested that seed inoculation

in conjunction with P and K, but not N, fertilizers will help to accelerate the growth of *A. ampliceps* and *A. salicina* in the framework of afforestation and reforestation programs especially in the arid lands of the Riyadh region.

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