**Synergistic effects of dual inoculation with rhizobia and arbuscular mycorrhizal fungi selected on peanut seedlings growth parameters**

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ABSTRACT

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| Plant inoculation with selected microorganisms remains a good alternative environment friendly. It minimizes the biodiversity loss caused by the excessive use of chemical fertilizers. The aim of this study is to evaluate the effect of dual inoculation with arbuscular mycorrhizal fungi (AMF) and rhizobia selected on growth parameters of peanut seedlings, in greenhouse conditions. The experiment was conducted in a greenhouse (5°23 N to 4°0 W) located at the University of Nangui Abrogoua (UNA) in Abidjan, Côte d’Ivoire during three months. Peanut seeds were surface scarified and pre-germinated on water agar in a petri dish. After three days of germination, the seedlings were transferred into plastic bags containing 1 kg of substrate. The fungal inoculum was added with 20g per seedling. As for the bacterial inoculum, 5 mL of bacterial suspension containing approximately 107 bacteria/mL was added dropwise around the rootlet after 5 days of sowing. Plants were watered regularly and their height was measured every two weeks. After three months of crop, plants were harvested ; plant growth, mycorrhizal and nodulation parameters were measured. Nodules were detached, counted, and weighted per plant. The aerial and root weights were determined after drying each compartment. Then, the mycorrhization parameters were determined after staining the roots. Results show better growth of inoculated plants compared to non-inoculated plants. Indeed, dual inoculation with both AMF and rhizobia has significantly improved yield paramaters such as the number and weight of pods and the nodules number. Significant increase in pods number from 1,44±0,53 to 2,33±0,87 (α = 0.05) was observed. Pods weight was significantly increased from 2,15±0,73 to 4,09±0,76 by dual inoculation. As for nodules number, a significant increase from 72,33±7,47 to 87,20±9,78 (α = 0.05) was also observed. So synergetic effects of both AMF and rhizobia are also beneficial for growth and yield parameters of peanut. |

*Keywords: Rhizobia, Arbuscular mycorrhizal fungi, arachis hypogea, dual inoculation, growth parameters, mycorrhization parameters.*

1. INTRODUCTION

Peanut is mainly grown in tropical and semi-arid areas where drought is a major problem due to scarce and unpredictable rainfall **(Rachaputia *et al.,* 2021).** Native to South America, peanut is cultivated in more than 100 countries and on more than 26.4 million hectares with an average productivity of 1.4 tonne/ha **(Ntare *et al.,* 2008)**. It is the 5th most important oilseed crop in the world after soybeans, cotton, rapeseed and sunflower.In 2012, global peanut production was estimated at 40.5 million/tonne **(FAOSTAT, 2012**). Africa alone provides around 27.4% of this production, mainly from Nigeria, Sudan and Senegal **(FAOSTAT, 2012).** Côte d’Ivoire is on the main producers of peanut in West Africa. Its production increased by around 5.6% from 2015 to 2023.

Peanut seeds are rich in fat with a content of 50 to 55%. They contain about 20 to 25% protein and are rich in minerals (phosphorus, calcium, magnesium, potassium) and vitamins **(Savage et Keenan, 1994)**. They also contain as much protein as meat, but unlike meat, it does not contain uric acid or cholesterol **(Pamplona- Rogers, 2006)** and is recommended in vegetarian diets. Peanut seeds are used as raw material in the oil and confectionery industries.

Peanut is a legume that helps maintain soil fertility by fixing approximately 552 mg of nitrogen per plant. Crop residues are also rich in protein and used for livestock feed and to produce fertilizers **(Asibuo *et al.,* 2008)**. The success of this plant in maintaining soil fertility lies in its ability to associate with atmospheric nitrogen-fixing bacteria. In fact, the peanut (*Arachis hypogaea L*.) is a legume able to associating with rhizobia. Thanks to this association, rhizobia help in restoring soil nitrogen through organs called nodules that they induce on the roots of their host plants **(Scheublin et Vander, 2006)**. In this symbiosis, rhizobia participate in the solubilization of nutrients via the production of organic acids, siderophores, and hormones. They also produce antibiotics and catalases that control pathogens **(Jaiswal *et al.,* 2021)**.

It’s also known that intercropping with legumes promotes soil fertility. The use of legumes associated with nitrogen-fixing bacteria (rhizobia) remains an effective means of restoring the fertility of degraded soils. Symbiotic nitrogen fixation by rhizobia in association with legumes is widely adopted in agriculture to reduce the excessive use of chemical nitrogen fertilizers. **(Abd-Alla *et al.,* 2023).**

Several studies have shown the beneficial effects of inoculating legumes with selected rhizobia. Thus, inoculation of cowpea with selected rhizobia increased grain yield **(Guimaraes *et al.,* 2019)**. Application of rhizobia associated with a recommended dose of nitrogen significantly improved peanut yield, growth parameters and seed quality as well as maximum uptake of major nutrients (nitrogen, phosphorus and potassium) **(Mondal *et al.,* 2020)**. The symbiosis between peanut plants and rhizobia is a major ecological process in the nitrogen cycle. This symbiosis allows peanut plants to colonize N-limited environments.

Peanut has the ability to also associate with arbuscular mycorrhizal fungi. In this symbiosis, AMF play a role in water and mineral nutrition, particularly in phosphate nutrition of plants **(Labidi *et al.,* 2012)**. In fact, AMF are soil microorganisms that can associate with most terrestrial plants. They contribute essential to soil fertility by increasing the capacity of plants to absorb nutrients such as P. AMF form mycorrhizae whose mycelia can extend to areas outside the rhizosphere and enlarge the root zone to absorb nutrients.

Mycorrhizal colonization of plant roots can expand the root absorption zone through the presence of external hyphae that grow and develop through the fine roots of plants **(Sukmawati *et al.,* 2021)**. Therefore, water and nutrients can be transported through hyphal tissues for uptake by plants **(Yaseen *et al.,* 2016)**.

It has been reported that AMF and phyto-beneficial rhizobacteria could interact synergistically to stimulate plant growth through a series of mechanisms that include enhancing nutrient acquisition and inhibiting plant fungal pathogens **(Artursson *et al.,* 2006)**. Thus, studies have also shown beneficial effects of this synergy on the growth parameters of legumes. Indeed **Ndoye *et al.,* 2015** presented the beneficial effects of dual inoculation with selected AMF and rhizobia on the growth parameters of *Acacia senegal* seedling.

However, in Côte d'Ivoire, very few studies have been carried out on the inoculation of peanuts with selected microorganisms, even fewer in Korhogo area**(Koffi *et al.,* 2018)**.

So, this study aims to evaluate the effects of dual inoculation with rhizobia and AMF selected on the growth parameters of peanut plants in a non-sterilized field soil of Côte d'Ivoire.

2. material and methods

**2.1 Soil sampling**

Soil used as substrate was collected in peanut field in Takali (9°25 N; 5°35 W) located in Korhogo area in northern Côte d’Ivoire. Soil collection was done at ten points of the plot, to obtain a representative composite soil sample of the targeted plot.

Physical and chemical parameters of this composite soil were determined at the Centre de Recherche en Océanographie (CRO) of Abidjan, Côte d’Ivoire. The pH (water) was measured in the supernatant of a soil / distilled water mixture in a ratio of 1:2.5. Organic and mineral matters content were determined according to **Moreno et al. (2001)**. Contents of total nitrogen (N) and phosphorus (P) were quantified according to **Bremner (1960) and Sherrell and Saunders (1966)**, respectively by atomic absorption spectrometer after digestion with concentrated sulfuric acid. Potassium (K) was analyzed by means of argon plasma ionization source mass spectrometer (ICP-MS) according to **Rao and Talluri (2007)** method.

**2.2 Rhizobial inoculum preparation**

Five (5) rhizobia strains selected for their efficiency on peanut plants growth, in controlled conditions, were used. They were isolated from peanut root nodules harvested in a field of Takali in Korhogo area (Cote d’Ivoire). These strains demonstrated their effectiveness and efficiency on peanut growth parameters. Each rhizobia strain was grown in YM (yeast mannitol) liquid medium **(Vincent, 1972)** under shaking at 150 rpm at 28°C for 2 to 4 days, until an OD approximately of 0.7 at 600 nm was obtained. This OD corresponds to a density of average 107 bacteria per mL. The five individual grown cultures were mixed in equal proportions (v/v/v/v/v) to obtain the rhizobial inoculum which contains approximately 107 bacteria per mL.

**2.3 Fungal inoculum preparation**

Fungal inoculum *G. aggregatum and G. etunicatum* were supplied by the Laboratoire Commun de Microbiologie (LCM) IRD/ISRA/UCAD of Dakar, Senegal. *G.* *aggregatum* (Schenck and Smith emend. Koske; DAOM 227 128) was isolated from Djignaki (Senegal) and *G.* *etunicatum* from Dijon (France). These strains were chosen for their performance in an efficiency test on many plants **(Kruger et al., 2012)**. They were isolated and multiplied on sterile soil, poor in phosphorus with maize as trap plant under greenhouse conditions. Three months after cultivation, roots were harvested and mycorrhizal inoculums were prepared as described by **Plenchette et al. (1989).** Each inoculum of fungi consists of sand, spores, hyphae and mycorrhizal root fragments. It contained an average of 40 spores per gram of soil and roots fragments with 80% of colonization **(Guissou et al., 1998)**. The mixed inoculum was obtained by a mixture of equivalent quantities of the two fungi and contained approximately the same spores number and infective propagules of each fungal species.

**2.4 Greenhouse inoculation test**

The inoculation experiment was conducted in a greenhouse (5°23 N to 4°0 W) located at the University of Nangui Abrogoua (UNA) in Abidjan, Côte d’Ivoire during three months (from October to December2016). The average temperature and humidity were 31.2°C and 38.80% respectively during the day and 26°C and 62.5% at night. Peanut seeds CNRA-ara 8-20 variety were provided by the Centre National de Recherche Agronomique d’Abidjan (CNRA). This variety has a short cycle of 90 days. Peanut seeds were surface-scarified in 70% calcium hypochlorite solution (CaCl2O2) for 8 min and rinsed several times with sterile water **(Gottardi and Nagl, 1998)**. They were then pre-germinated in Petri dishes containing 0.9% agar and incubated for 72 h at 28°C in the dark (covered with aluminum foil) in an oven. The pre-germinated seedlings were transferred into plastic bags containing about 1 kg of non-sterile soil moistened slightly with tap water.

Four (4) inoculation treatments were applied: inoculation with inoculum fungal (AMF), with rhizobia (R), dual inoculation with fungi and rhizobia (AMF+R) and control without inoculum. For each treatment, 9 replicates were performed in a completely randomized block. The fungal inoculation was done at sowing with 20 g of inoculum. Five (5) to seven (7) days after sowing, seedlings were inoculated with 5 mL of the bacterial suspension deposited drop by drop around the rootlet. Dual inoculation consists in inoculating seedlings with both 20 g of fungal inoculum and 5 mL of rhizobia inoculum. Plants were watered every day to maintain soil water content close to field capacity during 3 months. The height of plants was measured every two weeks during experimentation and after three months of crop, they were harvested. Shoot, root and total weights of peanut plants were obtained after drying at 70°C for 48 h. Before drying peanut roots, the fresh nodules and pods of each plant were detached, counted and weighted separately.

Frequency and intensity of mycorrhization of peanut roots were determined according to **Phillips and Hayman (1970)** method. For that, roots were previously rinsed with tap water and placed in tubes containing 10% KOH. The tubes were then boiled in a water bath at 90°C for 60 min. This step makes it possible to empty the cytoplasm content of the cells and to facilitate the coloration. The roots were rinsed abundantly with tap water to remove KOH, and then stained with 0.05% trypan blue which is brought to water bath at 80°C for 30 min. For each sample, root fragments of about 1 cm were mounted between slide and cover slide crushed in 20% glycerol and observed under a microscope. Estimation of root colonization by AMF was carried out using the method of **Trouvelot et al. (1986)** according to a rating system based on 6 classes. Mycorrhizal frequency (F %) and intensity (I %) were measured as follows: F % = (number of mycorrhizal fragments / total number of fragments observed) × 100 where (F %) is the frequency of mycorrhization reflecting the importance and the percentage of fragments of infected roots, with n as the total number of root fragments observed. I% = (95n5 + 70n4 + 30n3 + 5n2 + n1) / total number of fragments observed Where (I%) is the intensity of the cortex colonization expressing the portion of the cortex colonized with respect to the entire root system, with n5, n4, …, n1 as the number of fragments, respectively, denoted as 5, 4, …, 1.

**2.5 Data analysis**

The data obtained were analyzed using the XLSTAT 2010 software. The means values of different parameters were compared by the ANOVA according to the Student Newman Keuls test (p<0.05) for the inoculation test. Percentage data of root mycorrhizal colonization were arcsine transformed prior to analysis. Analyses were performed separately for each plant species.

3. results and discussion

**3.1 Results**

**3.1.1 Physical and chemical parameters**

Results of physical and chemical parameters are given in the following table

**Table 1. Soil physical and chemical characteristics**

|  |  |
| --- | --- |
| **Parameters** | **Values** |
| pH | 5.25 |
| Organic matters (%) | 4.23 |
| Mineral matters (%) | 2.53 |
| Total nitrogen (mg/kg) | 1.01 |
| Total phosphorus | 4.57 |
| Potassium (mg/kg) | 244.27 |

**3.1.2 Growth parameters of peanut seedling inoculated with rhizobia and AMF**

Results on height, shoot dry weight, root dry weight and total dry weight are given on table 2. These parameters except shoot dry weight, were improved by all treatments. Shoot height was significantly increased (α = 0.05) of 38,33±2,86 cm (in control) to 44,69±2,71 cm by the inoculation with rhizobia, with an increase rate of 43.69% compared to the non-inoculated control. However, no significant improvement in shoot dry weight (SDW), root dry weight (RDW) and total dry weight (TDW) was obtained. RDW tends to reduce under the treatments effect.

**Table 2:** **Height and biomass of peanut plants inoculated with both selected rhizobia and AMF**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Treatments** | **Height (cm)** | **SDW (g)** | **RDW (g)** | **TDW (g)** |
| **R** | 44,69±2,71b | 4,37±0,88a | 0,57±0,15a | 4,94±0,94a |
| **AMF** | 42,80±5,95ab | 4,29±0,97a | 0,70±0,12a | 4,99±1,03a |
| **R+AMF** | 42,82±4,63ab | 4,50±0,87a | 0,65±0,11a | 5,15±0,92a |
| **Control** | 38,33±2,86a | 4,00±0,94a | 0,71±0,14a | 4,72±1,05a |

Values following with the same letter in colunms are not significantly different according the Kruskal-Wallis test (p > 0,05). Mm: fungal inoculum; Rm: rhizobial inoculum; Rm+Mm: mixte inoculum of AMF and rhizobia. SDW: shoot dry weight, RDW: root dry weight, TDW: total dry weight.

**3.1.3 Effect of inoculation on peanut plant yield parameters**

An improvement of number and weight of peanut plant pods was observed with all treatments

The number and weight of pods varied respectively from 2.33 and 4.09 g (in the R+AMF treatment) to 1.44 and 2.15 g (in the control). The number of pods was significantly improved in (R+AMF) treatment (α = 0.05) with an increase rate of 61.80%. The pods weight was significantly improved with the same (R+AMF) treatment with a better increase rate of 90.23%.

AMF treatment has also significantly improved pod weight of 4,00±1,28g (AMF) to 2,15±0,73 g with a rate of 86.04% compared to the control.

**Table 3: number and weight pods of peanut plants inoculated with both selected rhizobia and AMF**

|  |  |  |
| --- | --- | --- |
| **Treatments** | **Pods number** | **Pods weight** |
| **R** | 1,78±0,67a | 2,88±0,81a |
| **AMF** | 2,22±0,97ab | 4,00±1,28b |
| **R+AMF** | 2,33±0,87b | 4,09±0,76b |
| **Control** | 1,44±0,53a | 2,15±0,73a |

Values following with the same letter in colunms are not significantly different according the Kruskal-Wallis test (p > 0,05). AMF: fungal inoculum; R: rhizobial inoculum; R+AMF: mixte inoculum of AMF and rhizobia.

**3.1.4 Effect of inoculation on peanut plant nodulation and mycorrhization parameters**

The number and weight of nodules varied respectively from 87.2 (in dual inoculation R + AMF) to 72.33 nodules (in the control) and from 0.20 (R treatment) to 0.17 g (in control). A significant improvement (α = 0.05) was observed in the two parameters. Indeed, nodules number was significantly improved in dual inoculation with R+AMF of 72,33±7,47 to 87,20±9,78 nodules, with an improvement rate of 20.56%. similar trend was observed with nodules weight, which increased of 0,17±0,01 to 0,20±0,06 g in R treatment, so an increase rate of 17.65% compared to the control. However no significant improvement was obtained in mycorrhization parameters following inoculation with rhizobia and/or AMF (Table 4).

**Table 4: Nodulation and mycorrhization parameters of peanut plants inoculated**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Nodules** |  | **Mycorrhization** |
| **Treatments** | **Number** | **Weight (g)** | **Frequency (%) Intensity (%)** |
| **R** | 81,89±8,33ab | 0,20±0,06b | 9,65±1,98a 0,20±0,03a |
| **AMF** | 78,78±6,94ab | 0,19±0,0ab | 10±2,89a 0.24±0,03a |
| **R+AMF** | 87,20±9,78b | 0,17±0,04ab | 9,80±2,30a 0,22±0,04a |
| **Control** | 72,33±7,47a | 0,17±0,01a | 9.44±2,54a 0.19±0,06a |

Values following with the same letter in colunms are not significantly different according the Kruskal-Wallis test (p > 0,05). AMF: fungal inoculum; R: rhizobial inoculum; R+AMF: mixte inoculum of CMA and rhizobia.

**3-2- Discussion**

In general, results present a better increase of parameters studied following to single or dual inoculation with rhizobia and/or AMF. However, under natural conditions, several factors including the presence of native microorganisms may alter or reduce the positive impact of the introduced inoculants on plant growth **(Ndoye et al., 2015)**. Results showed that inoculation of peanut seedlings with rhizobia improved significantly their height. Several studies on increasing of plant height due to rhizobial inoculation were reported. These results confirm once again the stimulatory effect of these symbionts, described by several authors. Indeed, **Bakhoum et al. (2012)** and **Ndoye et al. (2015)** demonstrated positive effects of rhizobia inoculation on the growth of Acacia senegal plants in non-sterile soils. Similar effects of rhizobia inoculation were also observed by **Sajid et al., 2011**, on peanut plants.

A significant effect was observed on pod weight with R+AMF and AMF treatments. This could be explained by the introduction of AMF and by the synergy between the two strains of AMF and rhizoia. Indeed, peanut is a legume that associates with rhizobia and co-inoculation with fungi can promote synergy between the two symbionts. Some rhizosphere bacteria work synergistically with mycorrhizae, promoting their growth and protection against abiotic and biotic stress **(Barea et al., 2002)**. In this present study, dual inoculation significantly improved the number and weight of pods as well as the number of nodules. This beneficial effect could be explained by better nitrogen, phosphate, water and mineral nutrition provided by both symbionts (rhizobia and fungi) **(Liu et al., 2015).**

Results also show that uninoculated plants are nodulated and mycorrhized as inoculated plants. This is be due to the natural occurrence of AMF and rhizobia present in the non-sterilized soil. Johnson (1998) also mentioned that the colonization of AMF in the untreated control was due to the presence of the native AMF. AMF and rhizobia are soil-borne microbes that play a major role in improvement of plant nutrient uptake and resistance to several abiotic stresses **(Sun et al., 2018)**.

Rhizobia and dual inoculation (R+AMF) improved significantly the weight and the number of nodules. Indeed, rhizobia are well known as organisms that improve biological nitrogen fixation (BNF) and consequently nodulation in leguminous **(Kahindi et al., 1997)**. This would result in a significant amount of nitrogen fixed in nodules due to rhizobia. The process of biological nitrogen fixation (BNF) requires a large amount of energy input and the necessary metabolic activities generating such as energy are highly depend on P availability. Plant inoculation with AMF or rhizobia can increase crop productivity under abiotic stress. Arbuscular mycorrhizal fungi are known for their beneficial effects even under stress conditions (Ndiaye et al., 2011). Also, a supply of both AMF and rhizobia more effectively improves the beneficial effects of this symbiosis which would undoubtedly be due to a P supply provided by the AMF. AMF contribute to plant growth by improving phosphate nutrition **(Dudhane et al., 2011)**. Many studies have shown that the metabolism of AM fungi provides an increased supply of P, which in turn improves the symbiotic performance of rhizobia **(Almeida *et al.,* 2000)**. The interaction between AMF and rhizobia influences not only colonization development but also N and P acquisition and competition for resources **(Artursson et al., 2006)**.Dual inoculation with AMF and rhizobia also promotes legume growth parameters **(Tajini et Drevon, 2012).**

Surprisingly, no synergistic effect of dual inoculation was observed on shoot and root weight and on plant height. Similar trends of dual inoculation were observed by **Ballesteros-Almanza et al. (2010)** and by **Franzini et al. (2010)**.

However, rhizobia application significantly improved the nodule weight of peanut plants.

4. Conclusion

Results shown the beneficial effects on inoculation with rhizobia or/and AMF on some growth and yields parameters of peanut plant. Single inoculation with rhizobia improves plant height and nodule weight. While single inoculation with AMF or dual inoculation both rhizobia and AMF improves number and weight pods. Dual inoculation also improved significantly nodule weight of peanut plant. So synergetic effects of both AMF and rhizobia are also beneficial for growth and yield parameters of peanut. These results confirm several studies which demonstrated beneficial effects of inocualtion with selected rhizobia or/and AMF on peanut plant growth parameters.

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