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In vitro study of macro and micro nutrients decline in MS medium on growth and development levels of *Acacia tortilis* (Forssk.) Hayne

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ABSTRACT

Acacia tortilis is one of the valuable species growing in the southern regions of Iran, which is highly considerable for a variety of reasons such as stabilization of quicksand, medicinal uses and ecological functions. Low germination capacity of seeds, hard rooting of cuttings, the value of aesthetics and multiple medicinal uses highlight the importance of further research to explore methods for easy and efficient propagation of this species *in vitro*. However, seeds of *A. tortilis* were obtained from Khazar Center of Forest seeds located in Koloudeh, Mazandaran Province, Iran and a hot water treatment for 15 seconds was used for seed dormancy breakage. Then, seeds were cultured aseptically in MS basal medium without growth regulators. In this work, to investigate the reaction of seeds to different variables in culture medium, various concentrations of macro and micro nutrients (0, 1, 1/2, 1/4) were studied. Briefly, total treatments (T) were 10 and each treatment was replicated 3 times, with 10 members. Finally, ANOVA and Duncan tests were used to analyze data. Results showed that the best method for seed sterilization was obtained though using $HgCl_2$ (0.5%) for 10 minutes and ethanol (70%) for one minute. In addition, results showed that the highest root and stem length, fresh and dry weight of stem and root belonged to T_2 (macro elements + 1/4 micro elements), while T_4 (macro elements + micro elements) was in the second order and the lowest values of the abovementioned parameters were found in T_8.

Key words: *Acacia tortilis*, Murashige and Skoog's medium, Seed culture, *In vitro* conditions.
Article type: Research Article.

INTRODUCTION

The Fabaceae trees are one of the foremost critical components of forest vegetation due to their financial, ecological and biological significance (Nabipour *et al*. 2015). The genus *Acacia* is one of the important genera of the family Fabaceae (Subfamily Mimosaceae) including approximately 1200 species in Australia, Africa, India, Iran and America (Golamian *et al*. 2015). *A. tortilis* is the umbrella thorn, also known as umbrella thorn acacia, a tree with a medium to large crown native to most of Africa, primarily to the Savanna and Sahel of Africa (especially the Somali peninsula and Sudan), but also occurring in the Middle East (Nandwani 1995; Tanabe & Honda 1999; Aziz *et al*. 2002). In extremely arid conditions, it may happen as a small and wiry bush. However, it grows up to 21 m in height and the tree carries leaves that develop to approximately 2.5 cm in length with between 4 and 10 pair of pinna each with up to 15 pairs of leaflets. Flowers are little and white, highly fragrant, and happen in tight clusters. Seeds are produced in pods which are flat and coiled into a springlike structure (Conesa-Sevilla 2011). *A. tortilis* has beneficial medicinal properties and therapeutic potential. For instance, it has been used for treatment of different diseases like skin sensitivity, cough and inflammatory response. Moreover, it is a well utilized plant by the local population where it found commercially as well as medicinally importance (Girijashankar 2011; Shahinozzaman *et al*. 2012). Their specific value in dry zones lies in their extraordinary resistance to warm, drought, salinity, alkalinity, floating sand, grazing and repeated cutting. Additionally, some of the *Acacia* species have significant values for re-plantation and reclamation of waste land (Vengadesan *et al*.

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2002; Ali *et al*. 2012). Most of these species develop in arid and semi-arid districts, where the average temperature is 40 to 45 °C in summer and less than 5 °C in winter (Yadav *et al*. 2013). Since they are the great sources of tannin, gum and timber, hence play a vital socio-economically role for the arrangement of furniture, dyestuff and as a nourishment- added substance (Vengadesan *et al*. 2000; Vengadesan *et al*. 2002). Interestingly, they have a particular function of nitrogen fixation in their root knots through symbiotic association with prokaryotic life forms. In this way, they provide staple protein-rich nourishment to the world's expanding population on one hand and contribute soil fertility by giving accessible nitrogen on the other hand. These processes are imperative for the keeping up the ecological system due to the fast changes in the environmental variables (Haliza *et al*. 2016).
Of all the components in nature, almost 40 are fundamental for living things (Hasanuzzaman *et al*. 2020). These components, which are fundamental for natural matter, are separated into two categories: macro- and micro-nutrients. Macro nutrient elements such as carbon (C), hydrogen (H), nitrogen (N), phosphorus (P), calcium (Ca), potassium (K), magnesium (Mg), etc., are required by plants in expansive amounts, regularly play a key role in the cytoplasmic structure of living cells. Conclusively, C, O, H and N, which together make up 95% of the entire plant weight, are the foremost vital components of protein (Saikat *et al*. 2016). These components, in conjunction with phosphorus, are the building blocks of numerous nucleic acids that store the genetic data of cells. Phosphorus is additionally required for energy conduction in cells (Haider *et al*. 2010). Micro nutrient elements such as boron (B), manganese (Mn), copper (Cu), zinc (Zn), molybdenum (Mo), cobalt (Co), vanadium (V), iron (Fe), chlorine (Cl), etc. are required by plants in very little amounts. These components play a basic part in the maintaining of the cell structure and performing special processes such as biological fixation of nitrogen, photosynthesis, construction of enzymes and their actuation, and tree fruiting (Rout *et al*, 2008). Noteworthy, each of the macro and micro nutrients in plants plays a special role and the deficiency or lack of one component can not be compensated by increscent of others, so all components have the same value physiologically (Khalafalla & Daffalla 2008; Javed *et al*. 2012). *Acacia* trees are confronting different abiotic stress problems such as drought, minerals disarrange, saltiness, temperature, water and losing their growth reproductive yields. In addition, other products of different importance develop as a incredible problem and huge challenge for their management. Micronutrients such as Zn, Fe, Cu, Mn, Mo, Br and Cl as well as macronutrients such as C, H, N, P, Ca, K and Mg are essentials for the ordinary development and biomolecular functions of all the plants. The evaluation and management of micronutrient status in soil–plants can secure the Fabaceous plants to grow ordinarily and keep their particular function of nitrogen fixation, indeed under abiotic stress conditions (Dhabhia *et al*. 2010). Soils in unfavorable environmental conditions (drought, saltiness, degradation through erosion, etc.) for the most part appear the lack of micronutrients which account for a wide gap in between the generation level of the nourishment crops including Fabaceous plants additionally requiring their prompt management (Hasanuzzaman *et al*. 2020). Since the growth rate of plants depends on the concentration of mineral elements in the soil or culture medium, by changing these elements, the optimal growth and development of each plant can be examined. In addition to examining the growth and development of a plant, the production of secondary metabolites as well as salinity-resistant plants can be examined. Micro propagation of tree species offers a rapid means to produce clonal planting stock for afforestation, woody biomass production and conservation of elite germplasm (Rout *et al*. 2008). Low germination of seeds under natural conditions because of having physical dormancy, hard rooting of cuttings, the value of aesthetics, and several medicinal utilizations are reasons for further research on their easier and multitudinous propagation in vitro (Dhabhai *et al.* 2013). The aim of this study was to investigate macro and micro element concentrations on the rate of obtained seedlings growth.

MATERIALS AND METHODS

Seed collection and dormancy breakage

Seeds of *Acacia tortilis* were obtained from Khazar Center of Forest seeds located in Koloudeh, Mazandaran Province, Iran. Then, seed dormancy breakage was conducted using hot water for 15 seconds (Nabipour *et al*. 2015).

Seed sterilization

Five treatments were considered for sterilization of *Acacia* seeds (Table 1). Noteworthy, first the kidneys were washed with two drops of Tipol and two drops of Twin 20, then rinsed with distilled water. In order to rinse thoroughly and reduce the adverse effects of disinfectants, the disinfected seeds were soaked in sterile distilled water for one hour.

Table 1. Sterilization of different treatments of *Acacia tortilis* seeds.

Treatment number	Sterilization treatments
1	NaClO 3% (10 min), Ethanol 70% (1 min)
2	NaClO 3% (15 min), Ethanol 70% (1 min)
3	$HgCl_2$ 0.3% (5 min), Ethanol 70% (1 min)
4	$HgCl_2$ 0.5% (5 min), Ethanol 70% (2 min)
5	$HgCl_2$ 0.5% (10 min), Ethanol 70% (1 min)

Culture medium and growth conditions

The Murashige and Skoog's medium (MS; Murashige & Skoog 1962) was used as basal culture medium which prepared by adding 3% sucrose as carbohydrate source and 0.8% agar as a solidifying agent. The seeds were cultured aseptically in MS basal medium without growth regulator. In this study, in order to investigate the reaction of seeds to changes in culture medium, different concentrations of macro and micro nutrients (0, 1, 1/2, 1/4) were used. Totally, 10 treatments were used, each with 3 replications and 10 observations. The pH of medium was adjusted to 5.8 before autoclaving at 121°C for 20 min at 150 kPa. Then, 30 mL of medium was poured into a culture jar and the lid was closed. All cultures were incubated in 16 h:8 h photoperiod under light intensity of $\mu E/m^2/s$ provided by cool, white and fluorescent light at 25 ± 2 °C with 55% relative humidity (Dhabhai *et al*. 2010). In this work, some properties including the number of seed germination, seed germination percent, stem and root length, wet and dry stem weight, wet and dry root weight and top/root ratio, were measured for each treatment and replications after 2 months. Then, the data were statistically analyzed (Hosseini Nasr 2016). Data analuses were performed using one way analysis of variance (ANOVA), and the means by Dunkan test with 95% probability.The program used for data analysis and for drawing graphs was SPSS statistical analysis software.

RESULTS AND DISCUSSION

Seed sterilization

Among 5 different sterilization protocols (Table 1) that were applied, the best sterilization protocol has been figured out to be the seed treatment with Tween 20 and $HgCl_2$ (5%) for 10 min followed by ethanol (70%) for 1 min that controlled the pollution (One-Way ANOVA test, $p < 0.01$, F = 41.143, Fig. 1). One of the most difficult and important issues in *in vitro* cultures is contamination control, so any research in this regard should be done under completely sterilized conditions, otherwise no results are obtained (Esna ashari & Zokaie 2014). In this study, no contamination was observed in T_5 ($HgCl_2$ 0.5% for 10 min and ethanol 70% for 1 min). Therefore, the mentioned treatment was used to sterilize all seeds. It seems that the use of ethanol kills surface microorganisms and improves the absorption of sterilizing substances. Yahia (2009) obtained similar result with explants of *A. tortilis* subspp. *spirocarpa*.

Germination

Comparison of treatments in terms of mean germination percentage and germination time by analysis of variance and Duncan tests shows that there is a statistically significant difference between all treatments at the statistical level of 1% (One- Way ANOVA test, Germination day, $p < 0.01$, F=23.867. Germination percentage, $p < 0.01$, F = 139.498, Fig. 2).

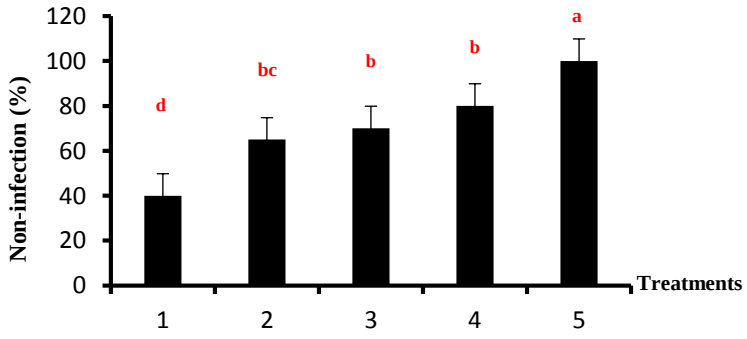

Fig. 1. Sterilization treatments for control of *Acacia tortilis* seed contamination by F test; The existence of at least one joint letter lacks a statistically significant difference.

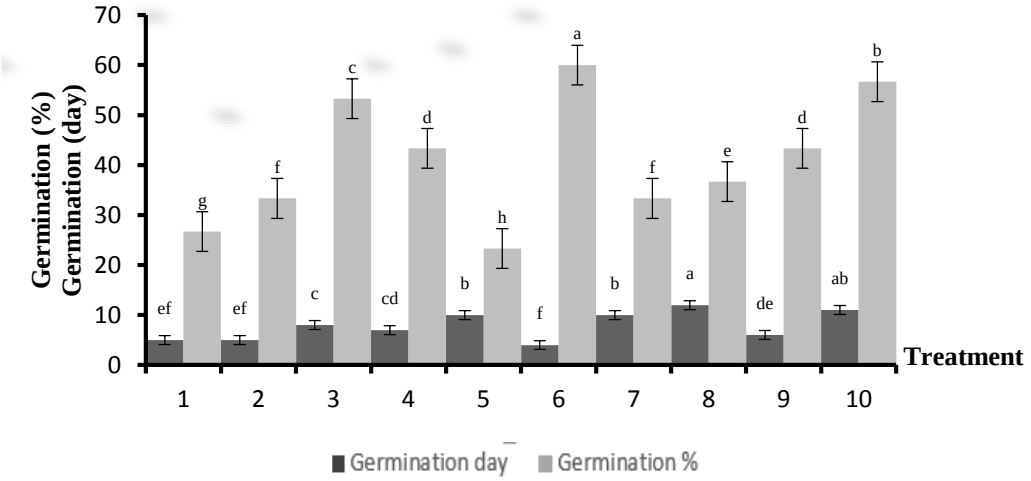

Fig. 2. Mean comparison of germination day and germination percentage of *Acacia tortilis* seeds in different treatments by Duncan test;The existence of at least one joint letter lacks a statistically significant difference.

Physical dormancy of seeds in the Fabaceae family is due to non-infiltration seed shells to water (Hasanuzzaman *et al*. 2020). Experimental results show that the impermeability of the seed coat in the legume family is due to a layer of sclerosing cells, breaking the coatings of these cells or mechanical pressure can cause water penetration and germination. For seeds of the leguminous family, pre-germination treatments are often used to infiltrate the shell and break dormancy, which is mainly soaking the seeds in hot water for a short time or scraping the seeds mechanically or chemically (Nabipour *et al*. 2015). In this study, for seed dormancy breakage hot water for 15 seconds was used. The use of hot water softens the outer shell of the seed, so the embryo will be able to begin to grow by absorbing water and oxygen. Germination time is one of the significant factors in seed growth, since delay in the germination, while is not economical, but also there is a possibility of seed damage in the soil or medium. In this study, T_6 (MS + ½ macro nutrients + ½ micro nutrients) exhibited the highest germination percentage (60%) in the shortest time (4 days). Notably, the seed germination is not related to the culture medium, but depends on factors such as germination energy, seed dormancy and related treatment, since the embryo uses seed storage for growth and not the culture medium. Similar results was obtained by Michael (1999) once working on *Acacia koa* seed germination under *in vitro* conditions.

Stem and root properties analysis

Comparison of treatments in terms of mean of stem and root length, wet and dry weights of stem and root by one-way analysis of variance and Duncan tests showed that there is a significant difference between all treatments at the statistical level of 1% (stem length: $p < 0.01$, F = 157.843, wet stem weight: $p < 0.01$, F = 14.228, dry stem weight: $p < 0.01$, F = 62.019. root length: $p < 0.01$, F = 156.916, wet root weight: $p < 0.05$, F = 15.827, dry root weight: $p < 0.01$, F = 23.843; Table 2). In this study, T_2 (macro elements + 1/4 micro elements) exhibited the maximum length, as well as wet and dry weights of the stem, while T_4 (macro elements + micro elements) was in the second order. The minimum stem length was found in T_8 (1/4 macro elements + micro elements) and the lowest wet and dry weights of stem belonged to T_1 (distilled water + agar). The maximum length, as well as the fresh and dry weights of root belonged T_4, while T_2 was placed in the second order. The lowest root length, wet and dry weights were observed in T_8. The results of the study of stem length, wet and dry weights of stem and root, show that T_2 (MS + macro nutrients + micro nutrients) has the longest stem and root length, while T_4 (MS + macro nutrients + 1/4 micro nutrients) has more wet and dry weights, indicating that the diameter of the stem and root is higher in the T_4, that is also more ecologically desirable. Decreasing the concentration of microelements probably caused a better and more absorption of macro nutrients (Esna ashari & Zokaie 2014). So, T_2 produced more stem and root volumes than other treatments. Noteworthy in this study, the aerial to ground ratio was also examined as one of the factors in the study of normal seedlings. Comparison of the mean aerial to ground ratio of treatments using one-way analysis of variance and Duncan analysis showed that there is a statistically significant

difference between the means at the statistical level of one percent ($p < 0.01$, F = 6.241,). In this study, the highest ratio of aerial to ground part belonged to T_8 while the lowest to T_7 (Table 2).

Table 2. Mean comparison of measured variables for *Acacia tortilis* seedlings in treatments (T) based on Duncan test.

T. No.	Stem length (cm)	Wet stem weight (g)	Dry stem weight (g)	Root length (cm)	Wet root weight (g)	Dry root weight (g)	Top/Root ratio
1	2.4de ± 0.55	0.0355d ± 0.003	0.0222e ± 0.0005	2.7e ± 0.213	0.1484bc ± 0.110	0.0085bc ± 0.0006	0.89bc ± 0.055
2	11.1a ± 0.68	0.3666ab ± 0.058	0.0708ab ± 0.0016	18.1a ± 0.291	0.2213a ± 0.008	0.026a ± 0.004	0.62bc ± 0.031
3	4.8c ± 0.04	0.2109bc ± 0.006	0.0635bc ± 0.0001	7.8c ± 0.083	0.1627a ± 0.001	0.256a ± 0.0019	0.69bc ± 0.001
4	10.1a ± 0.12	0.4016a ± 0.067	0.0814a ± 0.0041	16.5a ± 1.144	0.2262a ± 0.226	0.0267a ± 0.0024	0.64bc ± 0.043
5	2.5de ± 0.27	0.0538cd ± 0.008	0.0231de ± 0.0018	2.7e ± 0.398	0.0479bc ± 0.011	0.0079bc ± 0.0015	0.94abc ± 0.0734
6	3.4d ± 0.24	0.0983cd ± 0.014	0.0353d ± 0.0015	5.1d ± 0.169	0.1543b ± 0.084	0.0143b ± 0.0014	0.87bc ± 0.109
7	6.8b ± 0.28	0.2741ab ± 0.026	0.0668b ± 0.0018	13b ± 0.852	0.209a ± 0.021	0.0257a ± 0.0024	0.55c ± 0.037
8	1.9e ± 0.13	0.0696cd ± 0.225	0.0264de ± 0.0032	1.8e ± 0.177	0.0245c ± 0.004	0.0038c ± 0.0007	1.35a ± 0.225
9	7.4b ± 0.14	0.2605ab ± 0.061	0.054c ± 0.0067	12.5b ± 0.291	0.1484a ± 0.025	0.0224a ± 0.0002	0.63bc ± 0.026
10	1.9e ± 0.01	0.0463d ± 0.016	0.0234de ± 0.0005	2.3e ± 0.129	0.0293c ± 0.005	0.0047c ± 0.0008	1.04ab ± 0.147

Fig. 3. Seedlings of *Acacia tortilis* Under *in vitro* condition in T_2 (A) and T_4 (B).

Fig. 4. Growing of *Acacia tortilis* seedlings after 2 months in T_2 (A), T_4 (B), T_7 (C) and T_9 (D).

Fig. 5. Growing of *Acacia tortilis* seedlings after 2 months in T_1(A), T_5 (B), T_8 (C) and T_{10} (D).

Typically, seedlings with high root volume are preferred to seedlings with well-developed aerial parts and weak roots, because it will have a higher performance both in better establishment in natural conditions and in absorbing more water and food (Hasanuzzaman *et al*. 2020). In this study, T_8 has the highest value of the aerial to ground ratio (1.35), exhibiting low root growth, and the relatively ideal value of the intended characteristic belongs to T_2 (0.62). Numerous factors are involved in the production of root type and volume, including the type of culture medium, nutrients, types of hormones used in the culture medium, etc. It is worth mentioning that the use of suitable seedlings for different areas with different ecological conditions is a very important issue that by knowing this point, the desired seedlings can be produced (Hasanuzzaman *et al*. 2020).

CONCLUSION

The study of *Acacia* trees is important in many ways. These trees are valuable species that are very important in various aspects of the environment, genetic resources and medicine. In addition, the wood of these trees has considerable strength due to its tannin and gum production. The present study emphasizes the role of macronutrients and micronutrients on the growth rate of *A. tortilis* seedlings. Nutrients are very effective in producing seedlings of acceptable quality as well as suitable for different regions and specific ecological conditions. To further emphasize the results obtained and the adaptation of seedlings produced under *in vivo* conditions, it is suggested to conduct more studies.

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