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Biology-Botany

In vitro germination and seedling formation of *Plantago tomentosa* Lam. (Plantaginaceae): influence of concentrations of the MS medium

Germinação e formação de plântulas de *Plantago tomentosa* Lam. (Plantaginaceae) *in vitro*: influência das concentrações do meio MS

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ABSTRACT

Plantago tomentosa Lam. (Plantaginaceae) is an herbaceous plant native to Brazil. It is widely used in folk medicine. The species has potential for use in the pharmaceutical and food industries due to its possible bioactive properties and the presence of mucilage in the seeds. The objective of this work was to investigate the influence of different concentrations of Murashige-Skoog (MS) medium on germination and seedling formation *in vitro* of *P. tomentosa*. The seeds were collected in Lajeado, disinfected, and inoculated in bottles with MS medium added to 7 g L^{-1} of agar and 30 g L^{-1} of sucrose, in four treatments: 25, 50, 75, and 100% of the medium concentration. The experimental design was completely randomized, consisting of 17 replications of ten seeds for each treatment. The evaluation of germination and formed seedlings was carried out every two days to determine the variables of germination percentage (GP), mean germination time (MGT), germination speed index (GSI), seedling formation percentage (SFP), mean seedling formation time (MSFT), and seedling formation speed index (SFSI). No significant difference was found between treatments for all the assessed variables. It is concluded that the use of a concentration of 25% of the MS medium is viable, as it promotes a high percentage of germination and seedling formation in a time interval similar to that of the other concentrations, with the advantage of using fewer reagents.

Keywords: Bioactive properties; Folk medicine; Seed propagation

RESUMO

Plantago tomentosa Lam. (Plantaginaceae) é uma planta herbácea nativa do Brasil amplamente utilizada na medicina popular. A espécie tem potencial de uso na indústria farmacêutica e alimentícia por suas

possíveis propriedades bioativas e presença de mucilagem nas sementes. O objetivo do estudo foi verificar a influência de diferentes concentrações de meio Murashige-Skoog (MS) na germinação e formação de plântulas *in vitro* de *P. tomentosa*. As sementes foram coletadas em Lajeado, desinfestadas e inoculadas em frascos com meio MS acrescido de 7 g L^{-1} de ágar e 30 g L^{-1} de sacarose, em quatro tratamentos: 25, 50, 75 e 100% da concentração do meio. O delineamento experimental foi inteiramente casualizado, composto por 17 repetições de dez sementes para cada tratamento. A avaliação da germinação e das plântulas foi realizada a cada dois dias para a determinação das variáveis de porcentagem de germinação (PG), tempo médio de germinação (TMG), índice de velocidade de germinação (IVG), porcentagem de formação de plântulas (PFP), tempo médio de formação de plântulas (TMFP) e índice de velocidade de formação de plântulas (IVFP). Não houve diferença significativa entre os tratamentos para todas as variáveis estudadas. Conclui-se que o uso da concentração de 25% do meio MS é viável, pois promoveu alto porcentual de germinação e formação de plântulas e em intervalo de tempo semelhante ao das demais concentrações, com a vantagem de utilizar menos reagentes.

Palavras-chave: Propriedades bioativas; Medicina popular; Propagação por sementes

1 INTRODUCTION

Plantago tomentosa Lam. (Plantaginaceae) is a perennial herbaceous plant native to Brazil, occurring in the South and Southeast regions (except Espírito Santo) (Souza & Hassemer, 2015; Hassemer, 2020). The species is not endemic to the Brazilian territory, so it can be found in Argentina, Uruguay, Paraguay, Peru, and Bolivia, with a preference for dry and sunny fields and sandy and humid soils (Rahn, 1974; Hefler et al., 2011). Popularly known as transagem, tanchagem, or língua de vaca, it has tomentose leaves, arranged in a rosette and with indistinct petiole from the blade, spike inflorescences, and pixie-type dried fruits, containing three seeds each, with a wrinkled testa (Hefler et al., 2011; Moreira & Bragança, 2011; Hassemer, 2020).

The leaves or the whole *P. tomentosa* plant is reported to be used in folk medicine, both in southern Brazil and in Argentina, as an infusion, decoction, macerate, syrup, or dye, for the treatment of urinary tract infection, cystitis, inflammation, and sore throat or body aches (Vendruscolo & Mentz, 2006; Rodina et al., 2008; Merétika et al., 2010; Bolson et al., 2015). Furthermore, Ji et al. (2019) report that species of the *Plantago* genus are also traditionally used in Chinese medicine and that they have important biological activities such as immune system modulators, antioxidants, hypolipidemic

and hypoglycemic, intestinal regulation, anti-cancer, anti-inflammatory, among others. These properties probably derive from their polysaccharides. Also noteworthy is the seed mucilage that can be used in industry, with emphasis on the food industry, with possible dietary advantages over polymers of non-natural origin currently used (Soukolis, Gaiani, & Hoffmann et al., 2018; Noshad et al., 2021).

Experiments with extracts obtained from *P. tomentosa* show the biological activities, such as antioxidant and antibacterial. Palavicini et al. (2022) tested seed extracts on *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella choleraesuis*, obtaining low minimal inhibitory concentrations. Similarly, Duarte et al. (2002) observed the activity of plant extracts on *Staphylococcus aureus* and *Escherichia coli*, and on *Salmonella typhimurium* and *Pseudomonas aeruginosa*. Pachla *et al.* (2017) demonstrated a healing effect of the extract on surgical incisions for ovariohysterectomy in female dogs.

Three other species of the genus, *P. australis* Lam., *P. major* L., and *P. lanceolata* L., are designated as Unconventional Food Plants (UFP). Their leaves can be used in salads, bread, and broths, while the seeds can be turned into flour or used as sago (Kinupp & Lorenzi, 2015). Due to the anatomical similarities of these species with *P. tomentosa*, it is speculated that it could also be used as a UFP in human food, as pointed out in the work by Amato-Lourenço et al. (2020).

Thus, for using the potential of this plant, experiments on the ideal conditions for its propagation are necessary. The only works carried out in this direction were those by Dousseau et al. (2008), in which the effects of light, substrate, and temperature on germination and emergence were tested. And even those of Ramos et al. (2002) who described the biomass production of the species with different spacings and arrangements of plants in beds.

It is also considered that one of the most common forms of its propagation is obtained through seeds and that several factors can affect its germination, such as temperature, light, oxygen, presence of nitrates in the medium, content, and

availability of seed water. The latter is directly linked to the osmotic potential of the seed relative to the medium − if the medium potential is lower, the seed will lose water instead of gaining it, which restricts germination (Bewley et al., 2013) and, such conditions need to be investigated. Thus, one of the alternatives not yet investigated is *in vitro* propagation. This method consists of the cultivation of plant tissues or cells in a specific synthetic nutrient medium, under asepsis and controlled conditions, allowing, among other advantages, to obtain plants free of contamination and viruses, rapid multiplication, genetic improvement and allows the study of growth and the development of plant species (Davies Jr. et al., 2018).

Among the nutritive media described, one of the most used is Murashige-Skoog (Murashige & Skoog, 1962). It is noteworthy, however, that the dilution of the medium may yield superior results in the *in vitro* culture than the use of the original concentration (Saad & Elshahed, 2012), so laboratory tests are necessary to make these adjustments. Thus, the objective of this work was to investigate the influence of different concentrations of Murashige-Skoog (MS) medium on *in vitro* germination and seedling formation *of P. tomentosa*.

2 MATERIAL AND METHODS

Spikes with mature fruits (dark green) of *P. tomentosa* were collected from plants located in the urban area (a clean grassy field in a residential area) of Lajeado, Rio Grande do Sul (Latitude: 29°25'58" S, Longitude: 51°57'38" W), between October and November 2018. The seeds were manually removed from the pixies on a laboratory bench and the experiment proceeded a few days later. In horizontal laminar flow, they were disinfected with 70% alcohol for one minute, followed by 1.5% sodium hypochlorite for 10 minutes and triple washed with autoclaved ultra-purified water.

The seeds were inoculated in flasks (5.5 cm in diameter, 7.5 cm in height) containing 30 mL of MS medium in addition to 7 g L^{-1} of agar and 30 g L^{-1} of sucrose, in the following treatments: T1 (25% of the medium concentration), T2 (50% of the medium

concentration), T3 (75% of the medium concentration) and T4 (100% of the medium concentration) (Table 1). The organic constituents of Murashige-Skoog medium at 25, 50 and 75% of its concentration were Glycine (0.5, 1 and 1.5 mg L^{-1}, respectively), Myo–inositol (50, 100 and 150 mg L^{-1}, respectively), Nicotinic acid (0.125, 0.25 and 0.375 mg L^{-1}, respectively), Pyridoxine (0.125, 0.25 and 0.375 mg L^{-1}, respectively) and Thiamine (0.025, 0.05 and 0.075 mg L^{-1}, respectively). The last treatment (100%) corresponded to 4.49 g L^{-1} of powdered MS medium (Sigma-Aldrich M5519), whose composition follows that stipulated by Murashige and Skoog (1962). In addition, the pH of the culture medium was adjusted to 5.8, autoclaved at 120°C, and 1 atm for 15 minutes. For each treatment, 170 seeds were distributed in 17 replications (one flask = one replication), corresponding to a total of 68 bottles and 680 seeds. The bottles were placed in a germination chamber at 20°C±1°C with a photoperiod of 16h for 15 days. Next, they were transferred to a growth room under controlled temperature conditions (25°C±3°C) and a photoperiod of 16h for 15 days for seedling development. The flasks were kept in a completely randomized design during the 30 days of cultivation.

Table 1 – Mineral salts in Murashige-Skoog (MS) medium at 25, 50 and 75% of its concentration, used for germination and in vitro seedling development of *P. tomentosa*

Macronutrients	**25%**	**50%**	**75%**	**Micronutrients**	**25%**	**50%**	**75%**
NH_4NO_3	412.5	825	1237.5	H_3BO_4	1.55	3.1	4.65
KNO_3	475	850	1325	$MnSO_4 \cdot 4H_2O$	5.575	11.15	16.725
$CaCl_2 \cdot 2H_2O$	110	220	330	$ZnSO_4 \cdot 4H_2O$	2.15	4.3	6.45
$MgSO_4 \cdot 7H_2O$	92.5	185	277.5	KI	0.208	0.415	0.623
KH_2PO_4	42.5	85	127.5	$Na_2MoO_4 \cdot 2H_2O$	0.063	0.125	0.188
NA_2-EDTA	9.325	18.65	27.975	$CuSO_4 \cdot 5H_2O$	0.0063	0.0125	0.0188
$Fe_2SO_4 \cdot 7H_2O$	9.33	18.65	27.98	$CoCl_2 \cdot 6H_2O$	0.0063	0.0125	0.0188

*Concentrations in mg L^{-1}

Source: adapted from Murashige & Skoog (1962)

The ideal temperature for seed germination was set at 20°C, based on preliminary tests and according to temperatures recommended by Dousseau et al. (2008) for *P. tomentosa*. In these same preliminary tests, the seedling formation was negatively affected at 20°C and showed better development at 25°C. This was also the temperature used by Dousseau et al. (2008) for the emergence test of seedlings of the species in different substrates (sand, subsoil earth, vermiculite, and Plantmax, and their mixtures at a 1:1 ratio). For this reason, we chose to use two temperatures in the present study, one for germination and the other for seedling formation.

The germinated seeds and formed seedlings were counted every two days, using the radicle protrusion of 1 mm as a parameter for germination, and for seedling formation, the presence of a primary root and at least two leaves, one of them with at least 0.5 cm. The physiologic concept of germination was used here, adapted from Taiz *et al.* (2017). The flasks contaminated by microorganisms (28%) were disregarded and the data obtained in the other flasks were tabulated and used to define the variables of the percentage of germination (GP), mean germination time (MGT), germination speed index (GSI), seedling formation percentage (SFP), mean seedling formation time (MSFT) and seedling formation speed index (SFSI). The average time variables were calculated according to Labouriau (1983), while the speed indices followed an adapted formula of Maguire (1962), being then submitted to analysis of variance (ANOVA), followed by Tukey test ($p < 0,5$) using the statistical program CoStat 6.45.

3 RESULTS AND DISCUSSIONS

No significant difference was observed among treatments for all analyzed variables (GP, SFP, MGT, MSFT, GSI, and SFSI). The mean values for each treatment were similar and the standard deviations were low (Table 2).

Table 2 – Means and standard deviations of germination percentage (GP) and seedling formation percentage (SFP), mean germination time (MGT) and mean seedling formation time (SFAT) in days, germination speed index (GSI), and seedling formation speed index (SFSI) from *in vitro Plantago tomentosa* Lam. seeds and seedlings in four different concentrations of MS medium ($p < 0.05$)

Treatment		GP (%)	SFP (%)	MFT (days)	MSFT (days)	GSI	SFSI
T1	Mean	78 ± 16.91	66 ± 21.81	15.68 ± 4.85	23.32 ± 3.93	0.6011 ± 0.1649	0.3062 ± 0.1096
T2	Mean	84 ± 13.42	76 ± 16.97	16.78 ± 5.47	23.34 ± 3.88	0.6290 ± 0.1777	0.3455 ± 0.0831
T3	Mean	91 ± 11.67	83 ± 20.62	16.61 ± 5.59	23.70 ± 2.70	0.6849 ± 0.1878	0.3795 ± 0.1112
T4	Mean	88 ± 10.92	83 ± 23.94	17.44 ± 7.44	23.80 ± 4.48	0.6933 ± 0.2438	0.3837 ± 0.1288

*T1 = 25% of the concentration of the MS medium; T2 = 50% 25% of the concentration of the MS medium; T3 = 75% of the concentration of the MS medium; T4 = 100% of the concentration of the MS medium. Organized by the authors (2022)

In general, during germination, seeds do not need an external supply of nutrients, as whatever is necessary for the development of the embryo will be supplied by the mobilization of reserves contained in the reserve tissues of the seed itself (Bewley et al., 2013; Bareke, 2018). Thus, Grattapaglia & Machado (1998) report that some species may reach a high germination rate even in the absence of nutrients in the culture medium. However, it is known that nitrate, most commonly in the form of KNO_3, may influence the germination of some species (Baskin & Baskin, 2014; Bareke, 2018). The MS medium presents nitrates in the KNO_3 and NH_4NO_3 components (Table 1) and, in the present study, the different concentrations of these nitrates between treatments did not stimulate or inhibit the observed variables.

During the disinfestation, in the triple-washing step, mucilage was formed in the seeds. The ability to produce this polysaccharide mucilage by the seed has been reported as a common characteristic of the *Plantago* genus (Cowley & Burton, 2021) and seems to be associated with the ability of species of the genus to occur in arid

or dry regions with nutrient-poor soils. This mucilage attracts and retains water, constituting an adaptation that allows survival in adverse environments (Teixeira et al., 2019; Cowley & Burton, 2021). In general, soils in arid and semi-arid areas have high salinity, low fertility, and low water availability to plants (Sá et al., 2013). The formation of mucilage allows the survival of these plants in these environments, as it maintains the osmotic potential very low (up to −0.54 MPa in tests with *Capsella bursa-pastoris* (L.) Medik., Brassicaceae). *C. bursa-pastoris* also produces mucilage and its ability to absorb water prevents its loss to the environment (Deng et al., 2014). Furthermore, Gadheri-Far et al. (2012) reported that *Plantago ovata* Forsk seeds did not have their germination percentage affected at NaCl concentrations of up to 200 mM and osmotic stress of approximately −1 MPa, showing the existence of resistance of seeds of the genus under such stress conditions.

In general, the high concentration of salts and sucrose in a medium tends to decrease its water potential, preventing the absorption of water by the seeds (Bewley et al., 2013). Resende et al. (2021) observed this phenomenon in the first days of *in vitro* germination of *Melocactus glaucescens* Buining & Brederoo seeds in MS medium at 100% compared to concentrations of 25 and 50%, even though at the end of the experiment there was no statistical difference between treatments. A similar result was observed by Albuquerque et al. (2016), in which the *in vitro* GP of *Comanthera curralensis* (Moldenke) L.R.Parra & Giul. was higher in MS medium at 50% concentration compared to 100%, while Braga et al. (2015) reported that *Pyrostegia venusta* (Ker Gawl.) Miers had significantly equal GP in seed cultivation in MS medium *in vitro* at concentrations of 50 and 100%. However, the authors did not report seed mucilage in any of the three species mentioned.

Comparatively, it can be assumed that because of the mucilage coverage, *P. tomentosa* seeds were able to absorb water and not lose it through osmosis, even at the highest concentrations of MS medium – which would explain the high germination percentage found even under these conditions. Indeed, germination begins with the

absorption of water from the medium (Bewley et al., 2013), which can be observed in this study as, during the first days after inoculation, an increase in seed size was observed.

According to Teixeira et al. (2019), there is evidence that seeds with higher mucilage production, such as *Plantago*, not only benefit from their low osmotic potential at germination but also less mortality and greater seedling development occur. This last characteristic was also observed in the present study, given the high percentage of seedling formation. Even at the highest concentrations of MS medium, the percentage of seedling formation was, on average, only 8.1% lower than the percentage of germinated seeds, that is, almost all germinated seeds formed seedlings.

After the initial absorption of water by the seed, endosperm reserves are mobilized to supply the metabolism of the developing embryo, with subsequent protrusion of the radicle and emergence of the cotyledons. It is at this stage that the plant will start photosynthesis and the absorption of nutrients from the medium for the formation of the first true leaves (Bewley et al., 2013). The formation of true leaves was characterized here by the formation of seedlings. In addition to the action of seed mucilage, the nutrients in the MS medium may have influenced this phenomenon in *P. tomentosa*. It is taken into account that the MS medium was originally described for the cultivation of *Nicotiana tabacum* (Murashige & Skoog, 1962), thus corresponding to the demand for minerals and vitamins for the development of this species and must be adapted to the cultivation of *P. tomentosa* as well as it is observed with other plant species.

The nutrients in the MS medium are also essentially important for plant metabolism, playing roles in the production of new molecules, also in photosynthesis, cellular respiration, osmoregulation, and hormonal responses, among others. Perhaps one of the most important elements is nitrogen, as 1 to 5% of the plant dry mass is made up of this element in the form of proteins, nucleic acids, chlorophyll, phytohormones, coenzymes, and secondary metabolites (Marschner, 2012; Taiz et al.,

2017). Nevertheless, Saad & Elshahed (2012) report that cobalt and iodine do not have their function specified in cell growth, while sodium and chlorine are not essential, but can be beneficial. However, despite their importance in plant metabolism, it is not possible to state that they were essential for the germination and formation of *P. tomentosa* seedlings, particularly because during germination and early development, the endosperm reserves are first consumed for this purpose. It is also important to note that the experiment conducted lasted only for 30 days.

As for the organic constituents (Table 1), although plants often produce their vitamins, the addition of thiamine, nicotinic acid, and pyridoxine can contribute to plant cell development. Myo-inositol is a carbohydrate that acts on cell division; glycine, on the other hand, participates as a source of amino acids and is not essential for the cultivation of all plant species (Saad & Elshahed, 2012). It is noteworthy, however, that these last elements would be more important in the cultivation of plant tissues, which is not the case in this study.

On the other hand, it is not possible to determine whether the components of the MS medium were essential for the germination and seedling formation of *P. tomentosa* in this work. But it is possible that, at concentrations lower than 25% of the medium or even in the total absence of nutrients, the variables studied remain similar. This possibility is reinforced when considering that *Plantago* genus plants occur naturally in soils poor in nutrients, with high salinity or heavy metals (Kuiper & Bos, 2012; Pol et al., 2021), which reinforces this possibility; however, further works studies with the species are needed so this statement can be made.

Also, it seems that the water uptake by the seedlings was not difficult, even at the highest concentrations of the medium, like what happened with the seeds. Pinhal et al. (2017) found a similar result for *Dipteryx alata* Vog. seeds maintained *in vitro*. In this case, no difference was found in the GP among the different concentrations of MS medium, but the seedlings had the SFP negatively affected at higher concentrations.

The authors report that the lower concentrations of the medium allow greater water absorption by the seedlings in formation, increasing the SFP, but this last factor differed from this work.

In addition to the water and nutrient availability, the temperature may have been another factor that influenced the evaluated variables, even if the seeds were kept in environments of 20ºC and 25ºC, respectively, for germination and seedling formation, for 15 days for each temperature. The evaluated variables were close to each other, because while the MGT was, on average, 16 days, the MSFT was 23 days. This shows that, on average, the seeds started to germinate one day after being removed from the germinator at a temperature of 20ºC. On the other hand, the seedling formed around half the time they were at 25 ºC.

Furthermore, the work of Dousseau et al. (2008) with *P. tomentosa* showed that the highest GP of the species was found at the 15-20ºC interval, and the highest GSI at 15 and 20ºC. This work, together with preliminary tests carried out before the beginning of this experiment, served as parameters to define the best temperatures for germination and seedling development used here. Furthermore, Gadheri-Far *et al.* (2012) pointed out that *P. ovata* had GP greater than 80% between 10 and 25ºC, but the highest germination index was around 20ºC. Thus, *P. tomentosa* seeds were able to germinate at both temperatures used in the experiment, while for the seedlings the temperature of 25ºC was probably crucial for their development.

4 CONCLUSIONS

The use of MS medium concentrations of 25, 50, 75, and 100% for *in vitro* cultivation of *Plantago tomentosa* seeds did not significantly influence on germination and seedling formation. Thus, the use of a concentration of 25% is recommended, as it showed a high percentage of germination and seedling formation in a time interval similar to that of the other concentrations, with the advantage of using fewer reagents.

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