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Enhancement of seed germination rate and growth of *Anagyris foetida* L.

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Abstract

Bean trefoil (*Anagyris foetida* L.), the only species within the *Anagyris* genus (L.) of the Fabaceae family in Algeria, demonstrates considerable promise for reforestation actions in arid and semi-arid areas throughout the Mediterranean basin. Nevertheless, a prominent obstacle impeding the successful establishment of the species is the hardness of its seeds, resulting in reduced germination rates and inconsistent seedling emergence. The objective of this research is to improve seed germination and seedling development in *A. foetida*. A total of 750 pods were randomly collected from a population including 13 individuals. From this sample, 100 intact pods and 100 seeds were chosen for measurement and weight assessment. Three different scarification techniques were utilized as pretreatments: chemical scarification, involving varying durations of immersion in 98% concentrated sulphuric acid; thermal scarification, involving 24 hours of exposure to a water bath set at 35°C; and mechanical scarification, achieved through the use of abrasive paper. Each treatment comprised four replicates of 50 seeds, while untreated seeds were utilized as controls. Germination and initial seedling development were assessed after 30 days of cultivation. Statistical analysis revealed significant differences ($p<0.001$) among pretreatments concerning germination and seedling emergence. The highest germination percentages were observed following 120 minutes and 150 minutes of sulphuric acid soaking, with 86% and 91.5% of germination, respectively. The chemical pretreatments demonstrated correlations with shoot and root growth, as well as collar diameter. In contrast, the control group exhibited notably low germination (5.5%), while mechanical scarification resulted in a 55% germination rate. Seedling survival rates ranged from 93.7% to 99.4%. The seeds of *A. foetida* predominantly exhibit physical dormancy attributed to their hard and impermeable seed coat. These findings could be valuable for the generative reproduction of the species, especially for producing plants for afforestation/reforestation programs.

Keywords

Anagyris foetida; Bioconservation; Dormancy; Fabaceae; Reforestation; Seed coat

Contents

1 Introduction

Anagyris foetida, commonly known as stinking bean trefoil, is a large shrub native to the Mediterranean basin with dense branches reaching heights of 2-3 m (Valtueña et al. 2012). Among Mediterranean flora, *A. foetida* possesses well-adapted physiological responses that contribute to an effective strategy for balanced water usage. It retains its leaves during autumn, winter, and spring, then becomes completely deciduous in summer, entering a dormant state and exhibiting high drought resistance (Avsar and Ok 2010). In this regard, stinking bean trefoil can be considered an indicator plant for the onset of dry seasons in a given region. *A. foetida* can adapt to a wide variety of soils and has been used as a fast-growing nitrogen-fixing pioneer species in large-scale native restoration of fire-damaged areas (Cardinale et al. 2008). *A. foetida* has trifoliate, alternate leaves and blooms from October-November to February-March. The flowers are papilionaceous, pendulous, odorless, greenish-yellow when young, and yellow when mature. The fruits are large pods containing several large seeds (Valtueña et al. 2007).

The leaves and bark of *A. foetida* are used in traditional medicine. Bellakhdar (1997) indicated toxic effects of *A. foetida* leaves. However, this shrub holds veterinary interest and treats mange in domestic animals (Innocenti et al. 2006). It is reported that leaves and fruits of *A. foetida* are commonly used in traditional medicine, for instance, in treating stomach pain and burns (Bnouham et al. 2006). Methanolic extracts of *A. foetida* when combined with amoxicillin have been reported to enhance activity against resistant strains of *E. coli* (Darwish and Aburjai 2010). Both leaves and fruits of *A. foetida* also exhibit nematicidal activity (Al-Banna et al. 2003). Tölü et al. (2012) reported that *A. foetida* leaves were the most consumed shrub species by goats after *Quercus coccifera* and *Genista anatoica*, respectively, among 17 different shrub species despite its coverage rate (6.5%). These same authors reported that its crude protein content ranged between 22.3% and 22.9% in a two-year study. Alatürk et al. (2014) also reported that the protein content of *A. foetida* leaves varied between 8.23% and 21.23% on a dry matter basis between two sampling periods in a one-year study. *A. foetida* would be an important source of forage for goats to meet their increased protein needs during early and mid-lactation (March to June) and would be used to treat their gastrointestinal problems such as nematode infections or gastrointestinal disorders (Akbag 2021).

A. foetida is classified on the red list of threatened species in France as well as on certain regional lists of protected plant species (Warlop 2006). In Algeria, there is not much information in the literature on the germination characteristics of this forest legume. Due to its rapid growth even in the poorest soils, which can improve nitrogen-poor soils, seedling production from seeds and their use in erosion control programs are very important. On the other hand, the abundance of seeds produced by this plant makes seed propagation an important method for reproduction. Generally, there is a higher percentage of seed presence around the mother plant; however, natural regeneration is low or very low (Houle 1995). Indeed, natural regeneration of *A. foetida* remains very low (2-5%), and existing populations are in total decline, especially with forest fires occurrence. In nature, regeneration occurs by supporting factors such as high or fluctuating temperatures, fire, frost, thaw, or passage through animal intestines (Nonogaki 2019). Generally, once the seed has become permeable, it will not become impermeable again, and germination could begin (Baskin et al. 2000).

Mechanisms controlling germination are significant in nature as they contribute to natural survival, dissemination, and species conservation (Kildisheva et al. 2020). Many seeds face challenges in germination, resulting in their propagation being affected by seed coat dormancy, leading to a low growth potential. Kheloufi et al. (2018) observed that seeds of most tree and shrub species in the *Fabaceae* family from arid and semi-arid regions cannot germinate rapidly when subjected to favorable germination conditions due to the water-impermeable seed coat. Very few studies have been conducted to evaluate seed germination in *A. foetida*, and the only two studies in this direction are those of Valtueña et al. (2008) from Spain and Avsar (2009) from Turkey. These authors confirmed that germination failure in *A. foetida* is attributed to seed coat hardness and that its seeds exhibit physical dormancy.

Seed scarification methods have been developed and modified over time to make them more practical and effective. Important methods of seed scarification include thermal scarification, freeze-thaw, mechanical scarification, and acid scarification. In this study, the effect of certain pre-treatments on the germination of *A. foetida* seeds was investigated. Thus, it aims to contribute both to the understanding of the germination characteristics of this species and to obtain useful information for the generative reproduction of the species. Finally, the production of seedlings would be intended for reforestation programs in arid and semi-arid regions.

2 Materials and methods

2.1 Study area and sampling

The seeds of *Anagyris foetida* used in this study were obtained from dry pods collected from 13 different shrubs of approximately the same size (approximately 2.5 m in height) on August 20, 2020, growing at an altitude of 134 m in a forested area located in Misserghin, an Algerian municipality in the province of Oran (latitude: 35°37' N; longitude: 0°44' W). The province of Oran is largely situated in the semi-arid Mediterranean climatic zone. In 2020, the year of our sampling, the following climatic conditions were recorded in the region: an average annual temperature of 18.4°C, an average annual maximum temperature of 24.2°C, an average annual minimum temperature of 13°C, total annual precipitation of 299.48 mm, a total of 69 rainy days, and an average annual humidity of 69%. The highest recorded temperature was 42.3°C on July 6, 2020, and the lowest recorded temperature was -2.4°C on January 6, 2020 (WCD Tutiempo 2020).

After harvesting, the pods were transported in paper bags to the Laboratory of Ecology and Environment, University of Batna 2 (Algeria), where they were sorted, described, and photographed (Figure 1). The pod sample was obtained by mixing the dry fruits from the 13 different shrubs. Seeds were manually extracted by opening the pods. A sample of 100 fruits (pods) and 100 intact seeds were measured and weighed.

The following morphological characteristics were recorded: length and width of pods; number of seeds per fruit; length, width, thickness, and moisture content of seeds. The moisture content of the seeds was obtained after drying at 130°C for 2 hours (ISTA 2015). The seeds were then stored in paper bags at 20-25°C for 4 months (until their use in March 2021).

Figure 1. *Anagyris foetida*, A- Sampling site; B- Beginning of flowering; C- Winter flowering; D- Fruiting; E- Some individuals in fruiting; F- Pods; G- Seeds; H- Vegetation recovery in autumn.

2.2 Experimental design and application of pre-treatments

A total of 4 replicates of 50 seeds for each pre-treatment were sown in plastic pots (Top diameter: 14 cm; Bottom diameter: 11 cm; Height: 17 cm) at a rate of 5 seeds per pot containing 1.5 kg of growing substrate and arranged in a completely randomized design with four replicates under greenhouse conditions at a temperature of 25-27 °C and a 16-hour photoperiod. The pots were monitored every three days to ensure that water saturation was maintained at 30%.

The growing substrate originates from the arid region of Biskra (Southeast Algeria) from a 5-hectare site intended for a reforestation program with *A. foetida* shrubs. Soil analysis results (at 25-30 cm depth) revealed a pH of 7.7; 0.17% total nitrogen (N); 182.8 mg/kg$_{soil}$ of phosphorus (P_2O_5); and 1.22 meq/kg$_{soil}$ of potassium. The soil has a loamy texture with an electrical conductivity of 2.58 ms/cm. This high electrical conductivity attributed to soil salinity is due to a high concentration of calcium ions (20.3 meq/100 g$_{soil}$) compared to Mg^{+2} (1.36 meq/100 g$_{soil}$), K+ (1.18 meq/100 g$_{soil}$), and Na^+ (1.25 meq/100 g_{soil}). The soil is classified as very calcareous with 53.1% total $CaCO_3$ (Mansouri et al. 2020).

Three pre-treatment methods were used, namely chemical scarification (30 min, 60 min, 90 min, 120 min, 150 min, and 180 min soaking in concentrated sulphuric acid 98%), thermal scarification (24-hour soaking in a water bath at a temperature of 35°C), and mechanical scarification (using 80-grit sandpaper) to physically create cracks on the seed surface. The control batch underwent no pre-treatment. Seed germination and initial seedling development were assessed after 30 days. The final germination percentage (FGP) was expressed as a percentage of the total number of seeds. The seedling survival percentage (SSP) is the percentage of the ratio of live seedlings to germinated seeds. Measurements of morphological parameters, including total shoot length (TSL), total root length (TRL), and collar diameter (CD), were recorded for 20 plants (n=20) from each treatment.

Analysis of variance (ANOVA) with one factor was conducted using version 9.0 of the SAS (Statistical Analysis System) software (2002). Mean separation was performed using Tukey's test for multiple comparisons, and the statistical significance threshold was set at 5%.

3 Results and discussion

3.1 Morphological characteristics

In this study, the pods of *A. foetida* have an average length of 14.5±2.29 cm and a width of 1.57±0.46 cm (Table 1). The average values of these variables are higher than those recorded by Avsar (2009) for *A. foetida* in Turkey (9.66 cm and 1.4 cm, respectively). The number of seeds per pod ranges from a minimum of 3 to a maximum of 9 units, with an average of 5.10±2.13 (Table 1). The average seed biomass per seed is 0.39±0.04 g. For a sample of 100 seeds, these seeds measure on average between 1.10 and 1.25 cm in length, 0.72 and 0.85 cm in width, and 0.47 and 0.67 cm in thickness, with an approximately normal distribution. The moisture content of the seeds was estimated at 6.19% according to the ISTA methodology (2015). The average weight of 1000 seeds was 423.8 g.

Table 1. Morphological characteristics of the pods and seeds of *Anagyris foetida* (n=100).

Variables	Mean	SD	min-max	CV (%)
Pod length (cm)	14.5	2.29	10.2-17.1	15.6
Pod width (cm)	1.57	0.46	0.79-2.04	29.4
Number of seeds per pod	5.10	2.13	3-9	41.8
Seed length (cm)	1.19	0.05	1.10-1.25	4.09
Seed width (cm)	0.78	0.04	0.72-0.85	5.54
Seed thickness (cm)	0.56	0.05	0.47-0.67	9.29
Seed weight (g)	0.39	0.04	0.33-0.48	11.2

SD: Standard deviation ; CV: Coefficient of Variation

3.2 Germination and seedling emergence

As shown in Table 2, the final germination percentages vary between 5.5% (untreated seeds, control) and 91.5% (150 min immersion in sulphuric acid). ANOVA showed that there was a statistically significant difference between pre-treatments in terms of germination percentage (F= 355; $p < 0.001$). Thus, the highest germination percentage was obtained with a 150-minute immersion in sulphuric acid (91.5%), followed by a 120-minute immersion (86%) and a 180-minute immersion (75%).

Table 2. Summary of the effects of different pre-treatments on various parameters related to seed germination and seedling emergence of *Anagyris foetida* after a period of 30 days.

Pre-treatments	FGP (%)	SSP %)	TSL (cm)	TRL (cm)	DC (cm)
Control (untreated)	5.5^{e}	93.7^{a}	7.28^{g}	10.3^{cd}	0.39^{bc}
30 min SA	9.5^{e}	95.8^{a}	6.85^{g}	10.5^{c}	0.41^{b}
60 min SA	30.5^{d}	94.5^{a}	9.57^{f}	9.80^{de}	0.40^{b}
90 min SA	63^{c}	99.2^{a}	11.3^{e}	9.16^{ef}	0.37^{c}
120 min SA	86^{a}	98.8^{a}	13.9^{c}	9.06^{f}	0.46^{a}
150 min SA	91.5^{a}	99.4^{a}	18.3^{b}	13.4^{b}	0.37^{c}
180 min SA	75^{b}	95.4^{a}	22.9^{a}	16.0^{a}	0.35^{d}
24 h water (35 °C)	28.5^{d}	98.4^{a}	10.5^{e}	10.6^{c}	0.40^{b}
Sand paper	55^{c}	99.1^{a}	12.6^{d}	10.4^{cd}	0.36^{cd}
F-value	**355*****	**0.57^{ns}**	**750*****	**242*****	**36.7*****

Means with the same letters are not significantly different at $p < 0.05$ (Tukey Test). SA: sulphuric acid; FGP: final germination percentage; SSP: seedling survival percentage; TSL: total shoot length; TRL: total root length; CD: collar diameter; ***: significant at the 0.01% level; ns: not significant.

Reports suggest that germination, seedling emergence, and survival of forest plants are constrained and limited by numerous factors that ultimately reduce yield and quality (Wallace and Clarkson 2019). Generally, the final germination percentage improved with an increase in soaking duration in sulphuric acid from 9.5% (30 min SA) to 91.5% (150 min AS) compared to untreated seeds (control) (5.5%). This indicates that extending the soaking duration in acid may have a more positive influence on FGP in *A. foetida*. In our previous work, it has been found that sulphuric acid solution is an appropriate chemical approach to promote germination in several legume species, including all Acacia species listed in Algeria (Kheloufi 2019). Concentrated sulphuric acid is known to be involved in breaking seed coat dormancy, enabling water uptake, enhancing amylase and protease activity, and thereby contributing to improved germination and other physiological indices (Kheloufi et al. 2019). Several studies have reported improved germination indices with sulphuric acid treatment (Kheloufi et al. 2020). The effect of sulphuric acid on promoting seed germination, seedling growth, and survival at the nursery stage could be due to the positive effect of acid on the seed coat, thus allowing easier water absorption and oxygen diffusion (Barton 1965; Rusdy 2017).

According to Table 2, a FGP of 55% was achieved after mechanical scarification with sandpaper. Indeed, this method was able to induce germination that exceeded the control germination by more than 10 times. Mechanical scarification using sandpapers has also been effective in some forest legumes such as *Prosopis ferox* (Baes et al. 2002), *Astragalus siliquosus* (Eisvand et al. 2006), *Rhynchosia capitata* (Ali et al. 2011), *Parkia biglobosa* (Okunlola et al. 2011), and *Ormosia arborea* (Gonçalves et al. 2011). However, the effectiveness of mechanical scarification may vary depending on the genus, species, and especially the size of the seed. The technique with sandpaper is very challenging to perform on a large, kilogram-weighted dense batch, especially for large-scale seedling production programs. Therefore, this method is only used for small quantities or if sulphuric acid is not available. Valtueña et al. (2008) indicated that sheep grazing on ripe fruits of *A. foetida* could enhance germination to 48%.

In this study, the germination percentage in *A. foetida* was 30.5% after a pre-treatment of 60 minutes immersion in sulphuric acid. This result is consistent with the result obtained by Avsar (2009) for *A. foetida* in Turkey, with 33.8% after the same pre-treatment. The germination percentage was higher with the prolongation of exposure time to sulphuric acid. However, the duration of 180 minutes decreased germination by 16% compared to the 150-minute immersion (Table 2). Differences may arise due to the concentration of the acid and the exposure time of the seeds to the acid, as seeds exposed for a long time easily deteriorate (Kheloufi and Mansouri 2017). These results revealed that the seeds of *A. foetida* mainly had physical dormancy due to a quite hard and impermeable seed coat. Similarly, it has been stated that seeds of forest legumes mainly have physical dormancy and that boiling water or acidic pre-treatments can be used to break physical dormancy (Kheloufi 2017; Kheloufi et al 2017). However, some seeds did not have severe dormancy. Indeed, the germination percentage obtained in the control group (5.5%) highlighted this fact. This also revealed that there are differences in terms of hardness or impermeability of the seed coat among the seeds. Thus, some seeds, which had no or relatively few problems with seed coat impermeability, germinated easily. This has been reported in various legume species: *Robinia pseudoacacia* (7.5%), *Leucaena leucocephala* (10%), *Styphnolobium japonicum* (12.5%), and *Erythrostemon gilliesii* (82.5%) after only 15 days of sowing, where some variability existed, both within the same seed lot and between seed lots (Kheloufi et al 2018).

Soaking *Anagyris foetida* seeds in 35 °C water for 24 hours induced germination of over 23% compared to the control (Table 2). Thermal scarification proved to be one of the most popular methods due to its simplicity and ease of implementation. Two main heating devices can be used in thermal scarification, namely the oven and hot water bath (water bath). The effectiveness of thermal scarification varies considerably depending on the heating devices, treatment times, and temperatures (Maldonado-Arciniegas et al. 2018). Thermal scarification with a hot water bath has not been effective on hard seeds of many species (Kimura and Islam 2012). Valtueña et al. (2008) reported that the germination rate of untreated seeds of *A. foetida* was zero, with no improvement following exposure to high temperatures.

Seed dormancy is determined by several factors, including dehydration, oxygen content, extreme temperatures, and soil acidity (Penfield 2017). These conditions are important for successful seedling production. The rest is determined by the internal qualities of the seeds themselves, including metabolism, the content of certain growth regulators, and the presence of certain germination inhibitors in the seed coat, which prevent and delay imbibition (which is the first step of the germination phase) (Bewley and Black 1994; Kheloufi et al. 2017). Furthermore, seed quality is a decisive characteristic for the geographical distribution and conservation of any plant species (Grossnickle and Ivetić 2017). In our study, ANOVA shows that there was no significant effect ($F= 0.57$; $p>0.05$) of the pre-treatments on the percentage of seedling survival (Table 2). Indeed, the survival percentage ranged from 93.7% to 99.4%, indicating that *A. foetida* seedlings are robust and vigorous, especially with large cotyledons that provide them with a large amount of energy reserves contained in the taproot due to its early development and establishment.

Figure 2. *Anagyris foetida*, A-B: Emergence of seedlings, C: Various sizes of seedlings after a 30-day period.

The length of the shoots and roots as well as the collar diameter of *A. foetida* for the 30-day period for the different pre-treatment methods are presented in Table 2. These three variables were significantly ($p<0.001$) higher for sulphuric acid (180 min and 150 min soaking). Immersion for 180 min in sulphuric acid resulted in the highest seedling length growth followed by seeds treated with 150 min. This is due to the early initiation of germination in this batch of seeds. Early germination is the result of cracks or lesions made on the seed that enhance water entry and gas exchange, leading to enzymatic hydrolysis and thus transforming the embryo into a seedling (Bradford 2017). Rapid seedling growth occurred because seedlings from scarified seeds had the advantage of absorbing a lot of water and initiating the photosynthesis process much faster than others. These results are consistent with those obtained by other researchers (Bonner 1984; Kamra, 1990; Pedrini et al. 2020). According to Rifna et al. (2019), seed germination is the most crucial step that affects the early growth and establishment of seedlings. Immersion for 30 min in sulphuric acid and the control showed slower growth, indicating delayed germination initiation. For collar diameter, an average of 0.39 cm was recorded for all pre-treatments with a higher diameter obtained in seedlings whose seed batch was treated with 120 min of sulphuric acid (Table 2). According to this study, it has also been found that the germination of *A. foetida* seeds is of epigeal type (Figure 2).

4 Conclusions

In this study, it was confirmed that the seed coat of *A. foetida* seeds is hard and impermeable, which is the main issue delaying its germination. Particularly, soaking seeds in concentrated sulphuric acid for 120 and 150 minutes resulted in remarkable germination rates of 86% and 91.5%, respectively. The employment of mechanical scarification demonstrated its viability, yielding a germination rate of 55%. While it may not represent the optimal pre-treatment, this approach notably surpassed the control, yielding germination rates exceeding tenfold. This underscores its potential as a pragmatic option for adoption in field settings by farmers or nursery workers, notably due to its circumvention of the necessity for controlled acid handling. These findings could be valuable for the generative reproduction of the species, especially for producing plants for afforestation/reforestation programs. *A. foetida* is highly valued due to its nutritional capacity, for its ability to fix atmospheric nitrogen, reduce fertilizer costs, and produce secondary metabolites with some medicinal properties.

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