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Regeneration of *Anthylliscytisoides L.* from cotyledonary nodes and effect of seeds morphology on the germination rate of spontaneous shrub in eastern region of Moroccan

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Abstract

Spontaneous shrub A. cytisoïdes L. is a species of forage used for livestock feed, due to its low digestibility and its high protein content. The objective of this study is to test the hypothesis that morphological dormancy and seed storage have an impact on the germination rate of *A. cytisoïdes L.* To achieve this goal, we have investigated two types of seeds, small and large seeds. In addition, we used three lots of seeds, during our trials: 2008, 2009 and 2010. Results show that morphological dormancy has a highly significant effect on the germination rates of *A. cytisoïdes L.*: 96% for large seeds and 28% for the small seeds. However, the storage has no significant effect on the emergence rate of seeds.*A. cytisoïdes L.* used for afforestation and reclamation of degraded Mediterranean areas, was successfully propagated from explants (cotyledonary nodes) for different soil and age tested.

1. Introduction

Although re-vegetation programs are usually undertaken with trees, under adverse environmental conditions, shrub species would be the choice material to develop shrub lands rather than attempt afforestation [1]. This attains a particular significance in semi-arid Mediterranean regions where the primary goal must be prevention of soil erosion. Production of plant material of the appropriate shrubs species to be used in re-vegetation has become critical [2]. In such environments, shrub species form part of the primary plant succession and are considered suitable candidates to be employed in the phytostabilisation of semiarid mine tailings [3]. Organic residues are commonly used as a plant nutrient source and as soil amendments in order to promote the phytoremediation process in such contaminated environments[4]. In this context, tissue culture technology offers a great potential for rapid and massive cloning of ecologically valuable Mediterranean species. To date, information regarding micro-propagation of shrubs of interest to recover degraded lands is still lacking.

Legumes are one of the botanical families who have greater interest in the Mediterranean area and particularlyin Eastern Morocco, due to their adaptation to arid and semi-arid environments. Their ability to grow on poor soils, their qualities recognized as feed plants as well as their interrelationship with herbivores contribute to the dispersion of seeds [5] by the faeces [6], or by ungulates of animals [7].

The fact that soil erosion and degradation processes currently affect much of the Mediterranean coastline, there is an urgent need to take action regarding the conservation of natural resources, regeneration and improving the fragile ecosystems in arid and semi-arid regions. To accomplish this, it would be important to rehabilitate *A. cytisoïdes L.*, which is currently in danger of disappearing because of its extensive use in eastern Morocco. Rehabilitation of certain indigenous legumes, including *A. cytisoïdes L.* and *Retama sphaerocarpaBoiss.*, has been the focus of several recent research studies. Many authors mentioned that legumes often have impermeable integuments that induce physiological dormancy and make germination of their seeds very difficult [8].
Some spontaneous legumes, including *A. cytisoïdes L.* should be the complementary study and eventual distribution. Similarly, give their considerable biomass of forage. The protection they offer against erosion, and their role as a pasture plant, [5] are to be highlighted.
A. cytisoïdes L. is also a forage species used for livestock feed. The food quality of the herbaceous stems is not higher, mainly because of its low digestibility. It has a number of important proteins [5]. Also, its great ecological value and soil improvement as have been demonstrated, in [9,10]. As reported in[11], the presence of impregnated teguments and physiological dormancy make seed emergence difficult, either, after scarification. Delayed seed germination leads to the accumulation of seed in the soil, forming a seed bank in Mediterranean ecosystems and this will decrease the propagation of *A. cytisoïdes L.* in field.
For our knowledge, few studies on morphological dormancy of *A. cytisoïdes L.* have been reported. Most studies have focused on lifting of exogenous dormancy, such as scarification and using sulfuric acid and hot water. All these pre-treatments have increased the germination ratesbut haven't been able to give positive results. Additionally, *A. cytisoïdes L.* is an endangered species in the Mediterranean area and specifically in the north-east of Morocco. Thus, there is a need to investigate the effect of morphological dormancy on the germination rate of this species. The objective of this study is to test the hypothesis that morphological dormancy and seed storage have effects on the germination of*A. cytisoïdes L.* Small and large seeds, and for this reasaon three lots (2008, 2009 and 2010) have been tested.*A. cytisoïdes L.* used for afforestation and reclamation of degraded Mediterranean areas, was successfully propagated from explants (cotyledonary nodes) for different soil and age tested.

2. Material and Methods

2.1. Plant material

2.1.1. Anthyllis cytisoïdes L.

*Anthyllis cytisoïdes L.*are shrubby plants that could growup to1.5 m, height. This species has a wide distribution in the Mediterranean parts of the northwest Africa and the southern and eastern Peninsula, including Balearic Islands, stretching in the south of the France and the eastern region of Morocco [10]. *A. cytisoïdes L.* is particularly disturbed and abandoned in fields and near to long roads [13], where they form low brushwood of variable density and floristic composition. Its root system penetrates to a depth of several meters in loose sediments or fissures in rocks. So, potentially, roots exploit a large volume of soil [14].

2.1.2.The origin of seed

A. cytisoïdes L. seeds used in this experiment were collected in July of 2008, 2009 and 2010, respectively, in the region of Guenfouda, province of Jerada (lat. 34°18'22.24"N, long. 2°10'45.89"W). They were, then, stored in plastic bottles at room temperature at the laboratory of the National Institute of Agriculture Research (INRA), Oujda, Morocco.

2.2. Seeds separations

The seeds of *A. cytisoïdes L.* are usually covered by hard and cutinised pericarp (envelope), which completely prevents the imbibitions of water and sometimes gas exchange. This process is the most suitable for legumes. To remove physical dormancy, we have used the method of seed decortication which has been carried out as follows:

- The extraction of *A. cytisoïdes L.* seeds has been carried out by threshing. We have used the abrasive paper to shell the pod and released the seed, threshing 100 cloves of *A. cytisoïdes L.*, by hand would take about 30 min, and the separation has been done manually. The shelled seeds have been used in the experiment and this method has already been reported in [15].
- After decortication, in order to exhibit the endogenous dormancy, we have proceeded to their separation and have noticed two types of seeds; there are small seeds and large seeds. To distinguish them, the shelled seeds have been soaked in running water for six hours, placed in clear plastic glasses on moistened filter papers as shown in Photos 1.

2.3. The effect of seed storage on germination rate

The impacts of seed storage on germination have been evaluated using three seed lots: 2008, 2009 and 2010. The choice of these three seed lots was due to their conservation in the laboratory for researches and investigations. In our experiments, we have used eight replicates for each seed lot and four replicates for each seed type.

Photo 1: shows explants grown for 25 days

2.4. Process

At first glance, we have prepared 24 clear plastic glasses; filter papers have been wetted and placed the seeds according to 25 seed/glass. Each type of seed had 4 repetitions. The glasses containing seeds have been covered by aluminium foil and placed inside the greenhouse, at INRA-Oujda, in order to offer a better germination conditions. The period of the trials has been conducted during15 days, from February 24th until March 11th, 2015. During this period, temperature recorded inside the greenhouse was between 10 °C to 35 °C. Observations have been recorded every day. This appearance of a radicle of more than one millimetre has been taken as criteria of germination. At the end of the period of experimentation (March 11th), we have recorded all the seeds that did not germinate.

2.5 Explants (cotyledonary nodes)

The cotyledonary nodes of 10 and 25-day-old seedlings of *A. cytisoïdes L.* were used as initial explants. They arrived at the height of 1 to 2 cm from the nodes. Seedlings were grown from germinated seeds in greenhouse conditions. Seeds harvested from plants grown in the field were peeled (remove the husk), soaked in running water for six hours to separate the small seeds, the latter causes a combined Morpho-physiological dormancy and then we put the seeds in plastic glass filter paper moisten, at room temperature (about 15 - 35 ° C) and under daylight. For the 10 day experiment, we used two types of soils: a) compost; b) mixture (50% compost and 50% clay). The seedlings were transferred after 10 days in tray pots of capacity of 77 plants, it is filled by one of soil type (photos 1and 2).

Photo 2: Cotylidonary node transplantation

2.5. Statistical analysis

The experimental design is a complete random block with two factors, the first being the effect of morphological dormancy on seed germination, which comprises two levels (seeds size). The second is the seed storage effect on germination that has three levels (seeds lots). This has been analysed by two-factor ANOVA using IBM-SPSS Statistics 21 software.In our data analyses, we have used the general Univariate linear model. Comparisons of averages have also been made by the Tukey multiple comparison tests [16].

3. Results and Discussion

3.1. Effect of morphological dormancy

Statistical analyses of ANOVA has revealed a very significant morphological seed dormancy effect on germination rates, with $\delta R_2 = 0{,}962$ (R_2 adjusted = 0,951) (Table 1). And mean comparison analyses show that the large seeds recorded the highest germination rate (96%) in contrast to small seeds (28%) at the end of the experiment (Table 2). This small percentage may be due to endogenous dormancy. The small seeds have not reached maturity, and most do not contain an embryo.

Table 1: Effect of morphological dormancy and viability of seed on germination rate.

Source	Sum of type III squares	Ddl	Average squares	F	Signification.
Corrected template	28315,333$^{\delta}$	5	5663,067	90,690	,000(*)
Ordered originally	92752,667	1	92752,667	1485,363	,000(*)
Viability of seed	**177,333**	**2**	**88,667**	**1,420**	**,268(*)**
Seed size	**28016,667**	**1**	**28016,667**	**448,665**	**,000(*)**
Viability seed x Seed size	**121,333**	**2**	**60,667**	**,972**	**,397(*)**
Error	1124,000	18	62,444		
Total	122192,000	24			
Total (corrected)	29439,333	23			

25 seeds / glass were placed. Each seed size had 4 replicates. δR_2 =, 962 (R_2 adjusted =, 951) $\gamma\alpha = 0.05$; *Significant.

Table 2: The comparison of the average of each type of seeds (germination in%).

Effect of seed morphological dormancy	Average	Standard error	Confidence interval 95%	
			Lower bound	Upper limit
Small seed	28,000	2,281	23,207	32,793
Large seed	96,333	2,281	91,541	101,126

Use of small and large seeds. Each glass contains 25 seeds of *A. cytisoid L.* each type of seed is repeated four times. The margin of error is Mean Square (Error) = 62.444; $\alpha = 0.05$.

The strong germination accumulated by *A. cytisoïdes L.,* (96%) could be explained by the exogenous physical dormancy release by seed decortication and morphological endogenous dormancy by the separation of small seeds. So, to have a uniform and rapid germination of *A. cytisoïdes L*., the small seeds must be peeled and separated before seedlings.

3.2. Effect of seed storage on germination

The Tukey Test showed that there is no significant effect of seed storage on germination rate (Table 3 and Figure 1). The three seed lots obtained the overall averages of 60%, 60.5% and 66%, respectively, for 2010, 2008 and 2009.

Table 3: The germination result Tukey test

The effect of seed storage on germination	N$^{\delta}$	Homogeneous subset
Seed of 2010	8	60,0000
Seed of 2008	8	60,5000
Seed of 2009	8	66,0000
Signification		,306

Seeds from lots collected in three different years: 2008, 2009 and 2010. Each glass contained 25 seeds of *A. cytisoïdes L.* and each seed size was repeated four times. The error term is mean square (error) = 62.444; δ Number of lots samples of the harmonic means = 8,000; γα = 0.05; ns = not significant."

Moreover, for the different seeds lots (2008, 2009 and 2010), the germination rate was the same for small and large seeds size (Figure 2). However, the large differences in germination rate between large and small size were recorder only after 7 days. This was recorded in the same tendency for all seeds lots. Hence, it confirms that seed germination of *A. cytisoïdes L.* is uniform for all seeds lots.

Endogenous dormancy includes the case of embryos where its physiological development is incomplete at the time of separation from the parent tree and which require some additional time to complete their development and be able to germinate. It also includes embryos that are morphologically mature at the time of seed dispersal or harvest, but are physiologically incapable of germination until some biochemical changes have occurred, which are still poorly understood. Authors [17] have reported that the small increase in the germination rate of *A. cytisoïdes L.* after scarification could be explained because of the physiological dormancy. For this species, delayed germination leads to the accumulation of seed in the soil, forming a seed bank in Mediterranean ecosystems that is essential for it propagation and existence in the environment

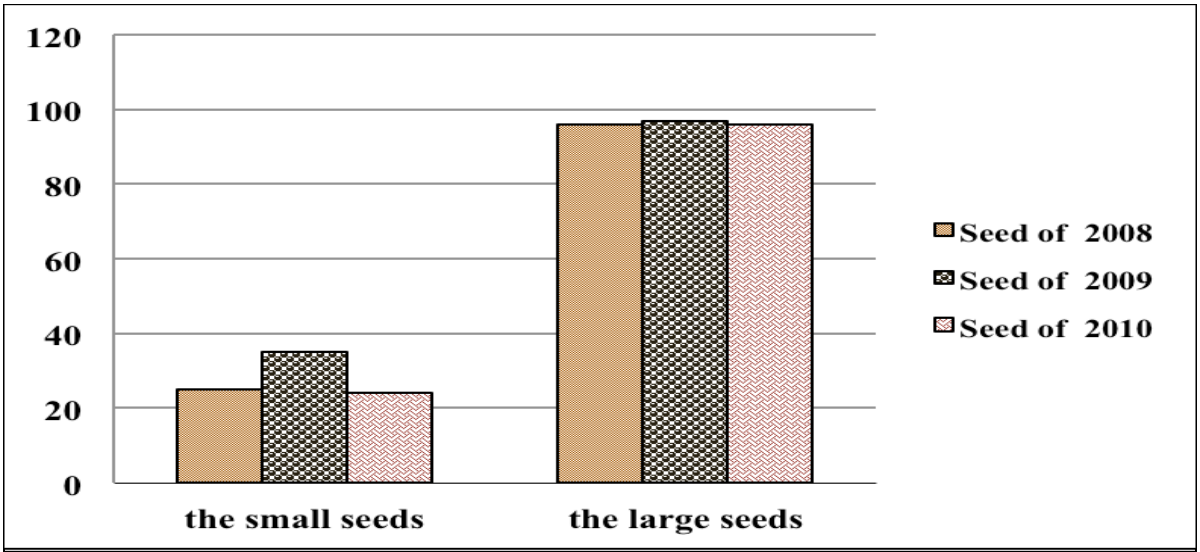
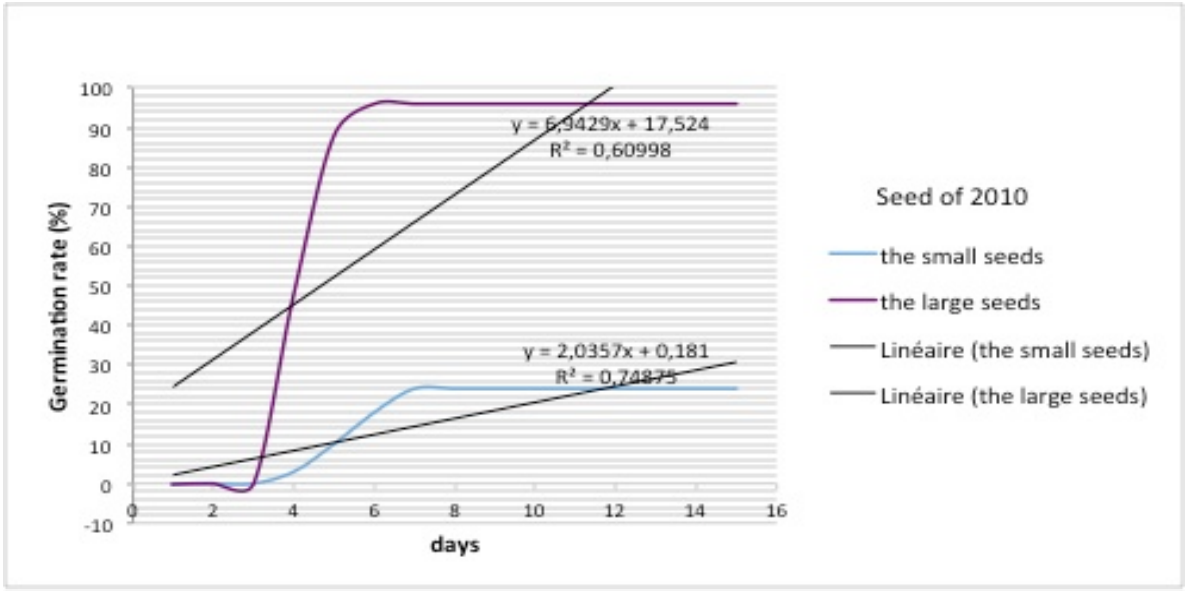
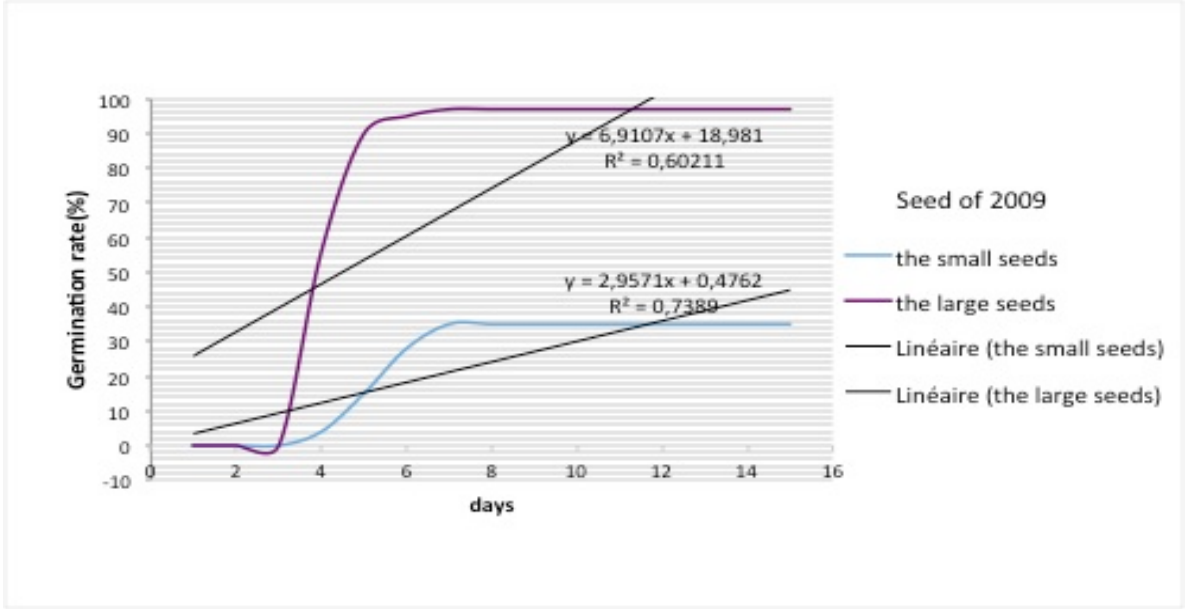


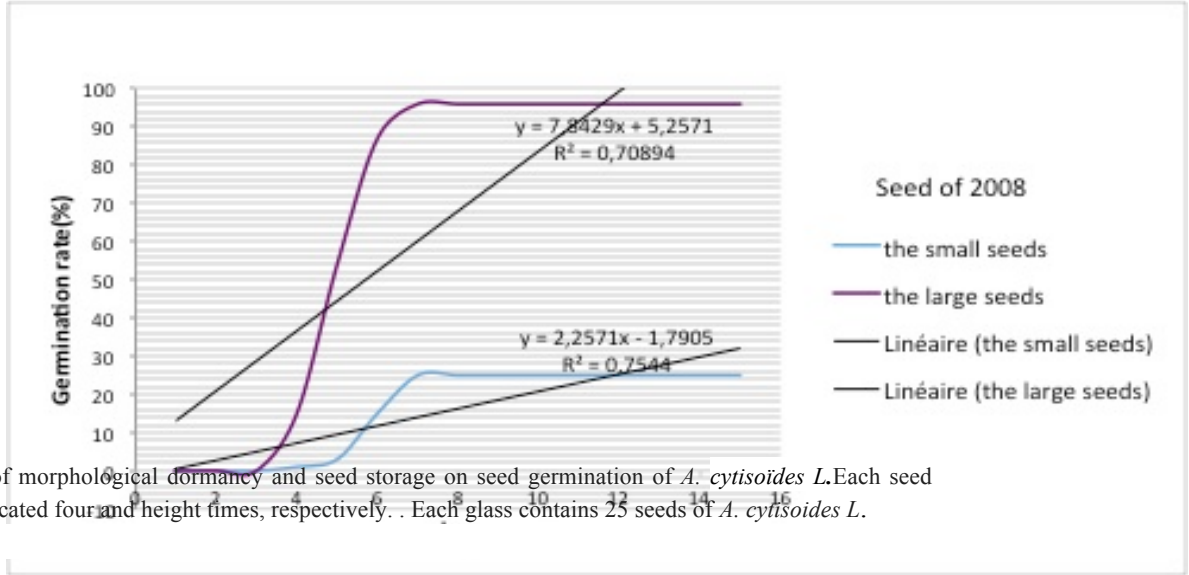
Figure 1: Diagram of *A. cytisoïdes L.* germination rate for different seeds size and lots**.** Each seed size and lot was replicated four and height times, respectively. . Each glass contains 25 seeds of *A. cytisoides L.*

In other side, our result shows that the small seeds of *A. cytisoïdes L.* do not germinate after mechanical treatment, perhaps due to the poor development of the embryo at the time of harvest as underlined [18]. Seeds with embryos, incompletely developed at the time of release or harvest, do not germinate until the embryos have reached maturity. This kind of morphological dormancy is known to occur in the seeds of others species, such as such as *Gingko biloba* [19]. In general, a period of pre-treatment with heat mist might be necessary to help the embryos, incompletely developed, to be able to germinate, as reported in [20].

Photo 3: shows the growth of explants aged 10 days in the soil compost.







gure 2: Effect of morphological dormancy and seed storage on seed germination of *A. cytisoïdes L.*Each seed e and lot was replicated four and height times, respectively. . Each glass contains 25 seeds of *A. cytisoides L*.

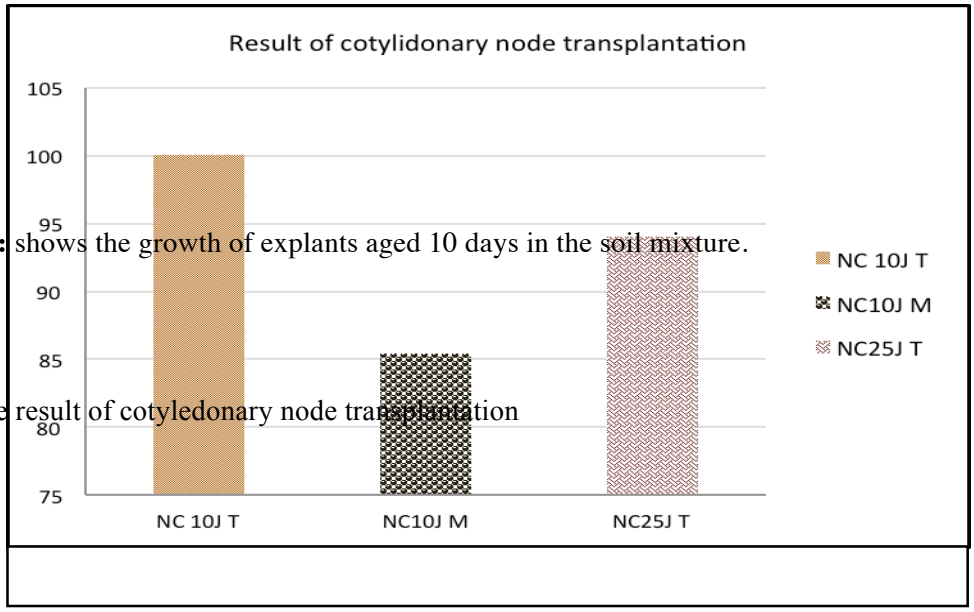


Photo 4: shows the growth of explants aged 10 days in the soil mixture.

Figure 3: shows the result of cotyledonary node transplantation

3.3 Success of transplantation

The results obtained showed a highly significant result on node transplantation. As shown in Figure (1) the 10-day cotyledonarynodes, compost soil (NC10JT) recorded a 100% successshown in Photo 3; the 25-days cotyledonary nodes, compost soil (NC10JT) accumulated 94% of the result and the 10-dayscotyledonary nodes, mixture soil (NC10JM) recorded 85.4% of result shown in Photo 4.

The bud development presents some differences, depending on the types of soils and growing conditions tested on cotyledonary nodes 10 and 25 days old soil compost, growth occurred after 5-10 days of culture. Shoots have proliferated from the buds and the formation of leaves was observed for these explants. Conversely, the cotyledonary nodes grown in soil mixture and 10 days old showed a slower development.

In its environment the *A. cytisoid L.* can grow in different types of soil, but preferably in plowed fields and / or abandoned crops.

Conclusion

This study has shown that the morphological aspect of *A. cytisoïdes L.* seeds has a significant effect on germination rate. This can be explained by the maturation or malformation of embryos in case of small size seeds. Results have also proved that storage effect does not affect the germination rate. Therefore, in order to have a uniform and rapid germination rate of *A. cytisoïdes L.*, it is necessary to dissect and separate the small seeds before seedlings. This will allow the rapid regeneration of seeds and the rehabilitation of rangelands. Few studies have been conducted on *A. cytisoïdes L* germination seeds. Consequently, furthers researches will be needed in order to understand genetic and physiological mechanisms behind the low germination rate of small size seeds and the weakness in propagation of *A. cytisoïdes L*in the field.

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