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Full Length Research Paper

In vitro germination and structure of hard seed testa of natural tetraploid *Trifolium pratense* L.

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Scarified and non-scarified seeds of natural tetraploid *Trifolium pratense* L. were germinated on *in vitro* and *ex vitro* germination media. The medium producing the best outcome was determined and germination media were compared. Additionally, testa of normal and hard seeds were examined. The media producing the best outcomes in both seed groups (scarified and non-scarified) were hormone-free MS medium, filter paper and soil respectively. Macrosclereid layer of normal seed testa was thicker than that of hard seed testa due to imbibitions. Whereas, epidermis walls in the macrosclereid layer of hard seed testa were thicker when compared to those of normal seed testa.

Key words: *Trifolium*, hard seed, *in vitro*, *ex vitro*, germination.

INTRODUCTION

Trifolium, a legume and a valuable feed plant, has been providing significant contributions to agricultural and animal production in U.S.A and Europe. Anatolia has been accepted as center of origin of *T. pratense* (Taylor and Smith, 1979). *Trifolium pratense* var. *pratense* found by Elçi (1982) in Tortum vicinity of Erzurum is natural tetraploid.

Megasporegenesis and megagametogenesis in natural tetraploid *T. pratense* L. were examined and it was concluded that failure in fertilization might affect the rate of seed setting as well as degeneration of embryo sacs and low rate of seed formation with respect to matured embryo sacs. When the development phases of this plant were examined from zygote to mature embryo, it was found out that there are many factors leading to ovul abortion (degeneration) and prevention of fertilization (unpublished data): 1. Failure of megaspore mother cell formation and the nucellus cells could be melted slowly. 2. There was a defect in the early stages of meiosis and the nucleolus could remain as a uniform tissue and then it may be melted. 3. Cell division started, but could not end, while the chromosomes were aborted the cell might probably have continued its development; defective division could happen in the subsequent developmental stages. On the other hand, the frequency of the megagametophyte formation was 20%, the development of the normal embryo being 5.8% shows that the normal developed male nuclei could not reach the megagametophyte or there might have been a defective fertilization (Algan and Bakar, 1996; 1997). In spite of an unproductive seed outcome due to the reasons mentioned above, hard seedness in Fabaceae family is a problem of natural tetraploid *T. pratense* L. as well.

Testa structure of natural tetraploid *T. pratense* L. was examined by Algan and Bakar (2000) and pollen and seed morphology was examined by Pınar et al. (2001), however, without any information on hard seed testa. Hard seed formation protects the embryo while it prevents imbibitions and germination. In general, hard seed formation in plants occur in order to protect the embryo until the existence of convenient conditions for germination. It has been stated that besides the environmental circumstances such as extremely high temperature during the maturation phase as well as the soil and climate characteristics, genetic factors also contribute to hard seed formation in Fabaceae family (Ünal, 2004; Elçi, 2005). Seeds are treated with different applications for eliminating this obstacle. While some researchers have used scarified seeds for increased germination rate (Phillips and Collins, 1979a; Myers et al., 1989; Maclean and Nowak, 1989; Grosser and Collins, 1984; Choo,

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1988; Konieczny, 1995), others have germinated seeds after a 30 s exposure in concentrated H_2SO_4 instead of using scarification paper (Bhojwani et al.,1984). No information has been reported regarding germination of natural tetraploid *T. pratense* L.

Researchers have germinated seeds of *Trifolium* L. species on different media such as B_5 (Gamborg et al.,1968), MS (Murashige and Skoog, 1962), SH (Schenk and Hilderandt, 1972) and SGL (Phillips and Collins, 1982; Phillips and Collins, 1979a; Bhojwani et al., 1984; Maclean and Nowak, 1989; Myers et al., 1989; Grosser and Collins, 1984; Choo, 1988; Cebrat et al., 1990a; Konieczny, 1995). Germinations on filter paper are generally preferred in cytogenetic and enzyme studies (Mohsin and Naqvi, 2000).

This study aimed to germinate the seeds of natural tetraploid *T. pratense* L. on *in vitro* germination medium and *ex vitro*, investigating the structure of hard seed testa which prevents germination.

MATERIAL AND METODS

Plant material

In this study, E2 type, 2n = 4x = 28 chromosome, natural tetraploid *Trifolium pratense* var. *pratense* L. (red clover) was used which was collected from "Tortum" vicinity of Erzurum (Turkey) by Elci (1982). The E2 type natural tetraploid *T. pratense* L. was grown in the experimentation gardens of Ankara University, Faculty of Science, and Department of Biology. Annual seeds are used in experiments.

Sterilization and culture conditions

To germinate the seeds *in vitro*, seeds were first sterilized in 96% ethanol for 1 min and then transferred to 10% sodium hypochlorite solution for 10 min (commercial sodium hypochlorite was used in sterilization process). Then seeds were rinsed 3 times in sterilized water. Non-scarifed seeds and seeds scarified with autoclaved sandpaper were germinated on hormone-free MS medium (Murashige and Skoog, 1962) and sterile water-wetted filter paper in plates. Non-scarifed seeds and scarified seeds were germinated in soil. All samples were incubated at 22 - 24°C in dark. Germination was controlled everyday. In a few days after germination, seedlings (with hypocotyl length of 2 cm) were incubated with 16/8 h photoperiod (irradiance of 42 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes).

Histological analysis

Seeds germinated on *in vitro* medium were classified as normal imbibed seeds, non-imbibed seeds and seeds with no change in color (hard seed). These seeds were fixed at 3% glutaraldehyde buffered with 0.1 M phosphate (pH 7.2) for 3 h at room temperature. They were then post-fixed with buffered (pH 7.2) 1% osmium tetraoxide for 3 h at room temperature. The material was dehydrated through a graduated ethanol series and embedded in Epon 812 (Luft 1961). Semi-thin sections were cut at 1.5 or 2 µm and stained with 1% methylene blue (0.5 - 1 min).

Statistical analysis

Data were subjected to one- way analysis of variance (ANOVA) and the differences among means were compared by Duncan's multiple-range test (Duncan, 1955). Each treatment was replicated three times and arranged in completely randomized design. Data given in percentages were subjected to arcsine transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS

Seed germination

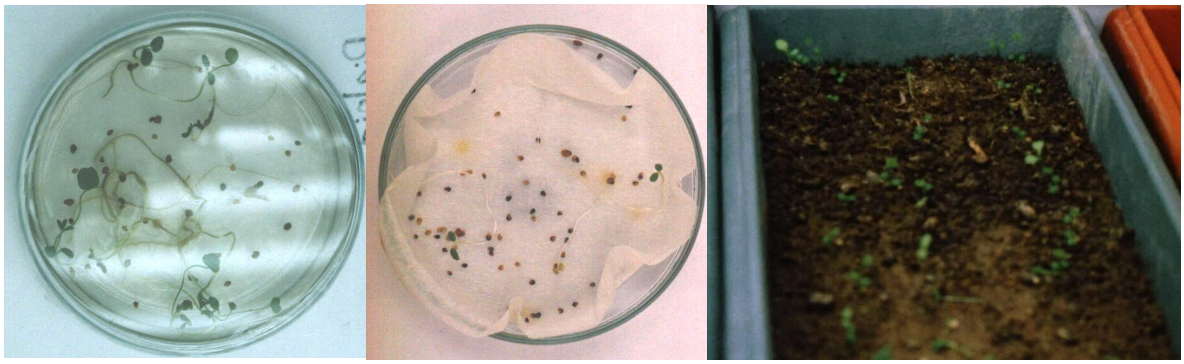
Non-scarified seeds were placed on hormon free MS medium and filter paper. After imbibitions, germination started at the 3rd day. Whereas, germination on soil took place within 5 - 7 days. The germination rates obtained on MS medium, filter paper and soil were 16.73, 13.51 and 4.5%, respectively. No imbibitions was observed in hard seeds on MS medium, filter paper as well as no change in colour (Figure 1). The percentage of the seeds which did not imbibed and hence did not germinated were 76.46 and 85.4% on MS medium and filter paper, respectively. Nevertheless, some imbibed seeds did not germinated as well (Tables 1 and 2).

Scarified seeds germinated at the rates of 90.15, 84.11 and 75% on MS medium, filter paper and soil, respectively. Germination started at the 3rd day following the placement of seeds on germination media (Figure 2) (Tables 1 and 2). Considering both scarified and non-scarified seeds, the medium with the highest germination rates was MS medium whereas soil was the medium with the lowest rates (Table 2).

Primer roots of the germinated 18 seeds on MS medium occured as calli at *in vitro* germination (Figure 3a). In the germination of one seed, cotyledons were observed first before primer root formation (Figure 3b). Primer root formation occurred in an abnormal manner in 23 seeds and no root growth was observed on hormone free MS medium. Germination was not completed in these seeds (Figure 3c). Abnormal root formation occured as well in the four seeds on wetted filter paper. An albino seedling was obtained among the germinated 1100 seeds on *in vitro* MS medium (Figure 3d).

Histology of seed testa

As a result of the measurements of hard seed testa, some macrosclereid cells were observed in normal imbibed seed testa with wide lumens extenting to the epidermis (Figure 4a). While average thickness of macrocsclereid layer of normal imbibed seed testa was 19.36 µm, the thickness of the layer of hard seed testa was 18 µm (Figure 4b). It was determined that macrosclereid layer in the hard seed testa was thinner than those of imbibed seeds. Outer cell wall thickness of macrocsclereid cells in normal seed testa and hard seed testa measured 7.57 and 11.62 µm, respectively (Table 3).



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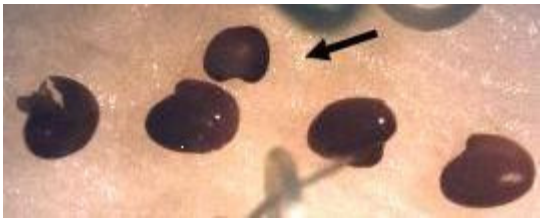

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Figure 1. a. Non-scarified seeds germinated on *in vitro* MS medium(left), filter paper(medium) and *ex vitro* (right). **b.** Hard seed (arrow) and imbibed seeds on filter paper.

Table 1. Germination percentages of non-scarified and scarified seeds on MS medium and filter paper.

Seeds	MS medium	Filter paper
Non-scarified seeds (%)		
Germinated	16.73±1.04	13.51±0.07
Non-germinated	83.27±0.15	86.49
Non-imbibed	76.46±0.15	85.4±0.03
Imbibed	6.81± 0.15	1.09±0.07
Scarified seeds (%)		
Germinated	90.15±0.17	84.11±0.04
Non-germinated	9.85±0.0	15.89±0.0
Non-imbibed	7.57±0.12	14.57±0.04
Imbibed	2.28±0.13	1.32±0.08

DISCUSSION

To increase the germination rate, Myers et al. (1989) scarified the seeds of *T. pratense*, while Grosser and Collins (1984) also scarified the seeds of *T. rubens* after surface sterilization like in our study. The scarified seeds were germinated on wetted sterile filter papers (Phillips and Collins, 1979a), hormone-free B5 medium (Gamborg et al., 1968) without saccarose (Maclean and Nowak 1989), MS medium (Konieczny, 1995), SGL medium (Collins and Phillips, 1982; Myers et al., 1989; Grosser and Collins, 1984; Choo, 1988); or on 0.9% agar (Cebrat et al., 1990a). However, no information on germination has been provided by the researchers. In our study, non-scarified and scarified seeds were germinated on hormone-free MS medium, filter papers and soil.

Germination rate was higher in the non-scarified seeds germinated on hormone-free MS medium when compared to those seeds on filter papers and soil. While some of the non-germinated seeds on hormone-free MS medium and on filter papers imbibed solution, failure in germination might have resulted from various abnormalities in seed development (Algan and Bakar, 1996,1997). Those seeds with no imbibitions were probably hard seeds. Considering the scarified seeds, germination rate was observed to be higher in MS medium with respect to filter paper and soil (Table 2). In line with our findings, Bringmann et al. (2002) have observed that scarification of two Kniphofia species (Asphodelaceae) had shortened germination duration and increased percentage of germination.

Abnormal germinations were lesser in the seeds on filter paper with respect to those seeds on *in vitro* medium. Cases like the formation but non-growth of primer root, occurence of cotyledons before primer root formation, etc, might have resulted from insufficient nutrients in *in vitro* medium as well as the abnormalities in seed development process (Algan and Bakar, 1996, 1997). Callus formation on *in vitro* medium might have occurred due to the contact of the root with meristematic and young cells with the medium. Albinism in plants is caused

Table 2. Germination of non-scarified and scarified seeds on MS medium, filter paper and soil.

Medium	Germination of non-scarified seeds (%)	Germination of scarified seeds (%)
MS medium	16.73 ± 1.04a	90.15 ± 0.17a
Filter paper	13.51 ± 0.07b	84.11 ± 0.04b
Soil	4.5 ± 0.23c	75.3 ± 0.15c

Values are means ± SE.
Mean followed by the same letter are not significantly different using Duncan multiple comparison test within different germination medium.

Figure 2. Scarified seeds germinated on *in vitro* MS medium (left), filter paper (medium) and soil (right).

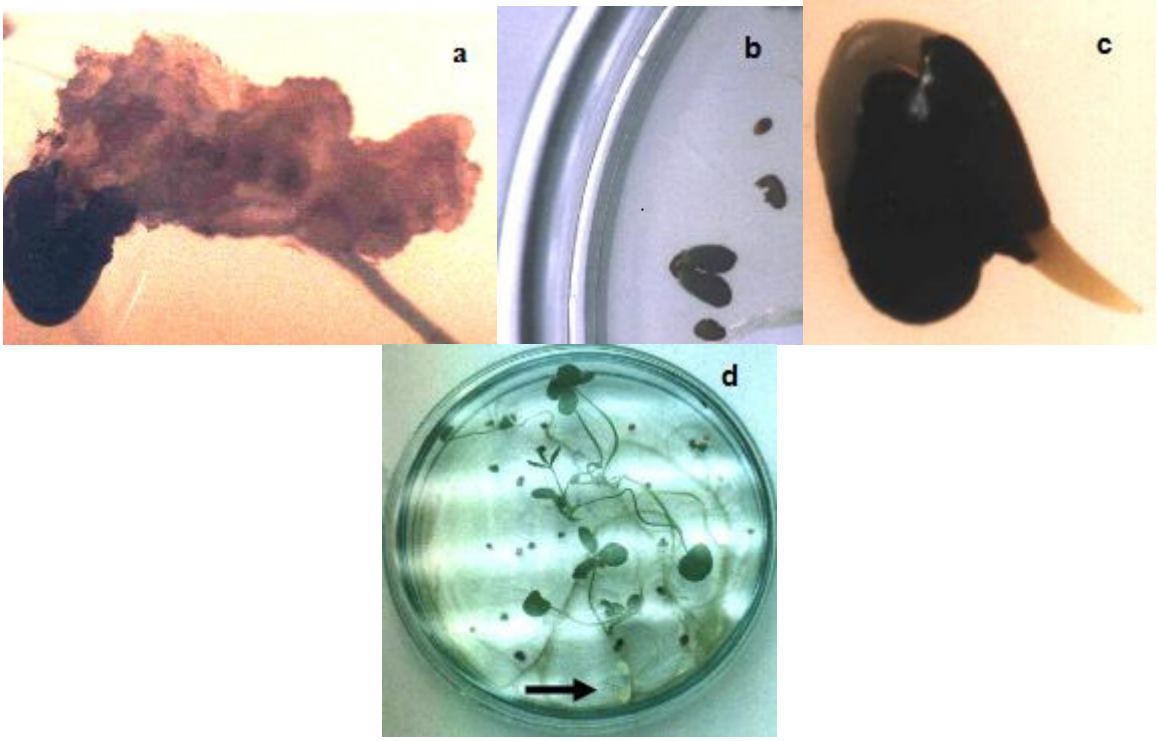
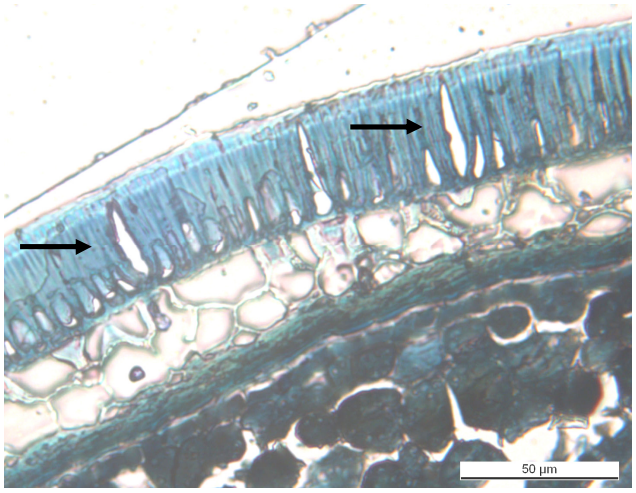


Figure 3. a. Root producing callus after germination on *in vitro* medium. **b.** Cotyledons at germinated seed before primer root formation. **c.** Abnormal germination (no root growth). **d.** Albino seedling (arrow).

Table 3. The measurements of natural tetraploid *T. pratense* seed testa.

Seed	Macrosclereid layer	Outside wall of macrosclereid cells
Soft seed	19.36± 0.17 µm	7.57±0.10 µm
Hard seed	18.03± 0.16 µm	11.62±0.04 µm



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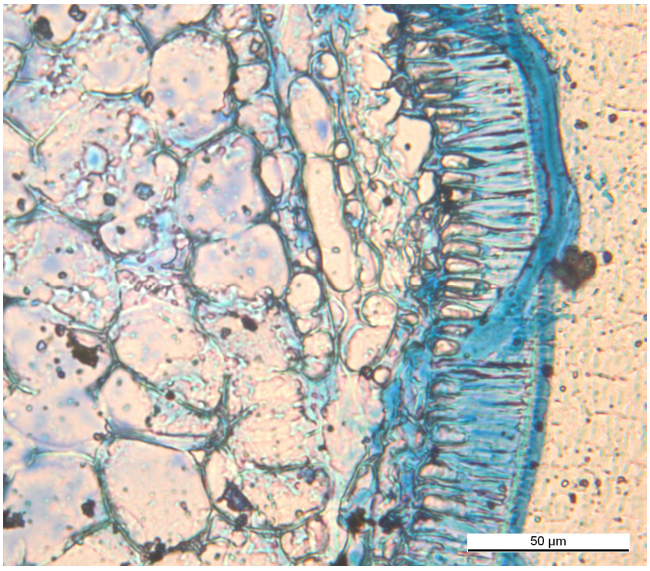

b

Figure 4. a. Normal testa (imbibed seed); lumens of macrosclereid cells extented to surface of the seed (arrows). **b.** Testa of hard seed (not-imbibed).

by the lack of a pigmentation. The albino plants are those that have a genetic mutation in their chlorophyll producing genes. However, in plants such deficiency is fatal because the missing pigment is chlorophyll. Without chlorophyll, the albino plant has no way to manufacture the food needed for survival and growth to maturity. In our experiments, albino individual could be observed through the sufficient conditions provided by the nutrient. Identification of one albino seedling per 1100 indicated the existence of albinism among this species with a probability of 0.09%. No details regarding such observations were mentioned in the studies of other researchers on *Trifolium* L.

In the testa of normal imbibed seeds (mostly, followed by germination), some macrosclereid cells were observed with wide lumens extending to seed surface. These cells are probably the weak zones facilitating imbibitions. No such cells were observed in the hard seed. The permeability of the testa can only be achieved via the cracks leaded by scarification.

Our findings reveal that percentage of germination among the seeds of natural tetraploid *T. pratense* is higher in *in vitro* conditions. Testa structure of hard seed needs to be considered in further studies of this plant. Our future studies also aim at examining hard seed testa and thin structure of the cells with wide lumen in imbibed seeds.

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