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In Vitro Pollen Germination and Pollen Tube Characteristics in Tetraploid Red Clover (*Trifolium pratense* L.)

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Abstract: The morphological characteristics, fertility and in vitro germination of natural tetraploid red clover (*Trifolium pratense* L.) pollen were examined as well as the characteristics of pollen tubes and callose formation. Pollen grains of red clover are tricolporate and usually the germination of pollen is monosporic. Among the pollen tubes that were formed under in vitro conditions, in addition to those that went through the normal growth process, poorly or non-grown pollen tubes were also observed. In addition, some abnormalities in the form of callose deposition were observed in the pollen tubes.

Key Words: Tetraploid, *Trifolium pratense* L., in vitro germination, pollen, red clover.

Tetraploid Çayır Üçgülü (*Trifolium pratense* L.)'nde İn Vitro Polen Çimlenmesi ve Polen Tüpünün Özellikleri

Özet: Doğal tetraploid çayır üçgülü (*Trifolium pratense* L.) polenlerinin morfolojik özellikleri, fertiliteleri ve in vitro ortamdaki çimlenmeleri ile polen tüplerinin özellikleri ve kalloz oluşumları incelenmiştir. Çayır üçgülü polenleri trikolporate ve polen çimlenmesi monosporiktir. İn vitro şartlarda oluşan polen tüplerinde normal gelişimle birlikte, zayıf veya gelişmeyen tüplere de rastlanmış ve bazı polen tüplerinde kalloz birikimi gibi anormallikler gözlenmiştir.

Anahtar Sözcükler: Tetraploid, *Trifolium pratense* L., in vitro çimlenme, çiçek tozu, Çayırüçgülü.

Introduction

In Turkey, a country where agricultural and livestock industries have an important role, the need for forage plants has been increasing gradually. The amount of forage plants used in agriculture today is limited. However, many research results and studies to date show that the best samples of various forage plants are parts of the natural plant covers of various regions in Turkey. An example is the legume *Trifolium pratense* L. (Red clover).

Tetraploid *T. pratense*, which does not naturally exist in any other regions of the world but grows naturally in Turkey, is a forage plant with high economic value due to its tetraploid plant characteristics and its superior protein capacity. Despite the agreement of international researchers that Anatolia is its centre of origin (Taylor & Chen, 1988), sufficient attention is not paid by our agricultural institutions with regard to *T. pratense* cultivation. Unfortunately, tetraploid red clover produces a very small amount of seed. This may be due to several

reasons. There may be some difficulties in microspore and megaspore formation due to some abnormalities in the male and female gametophyte or due to difficulties in fertilization and post-fertilization stages. Therefore, many researchers have dealt with the growth of pollen tubes in the style after the pollination in this plant (Evans, 1962; Mackiewicz, 1965; Chen & Gibson, 1972; Kazimierski et al., 1972). Pollen germination and the growth of pollen tubes are, in principle, necessary for fertilization and seed formation in flowering plants. Studies on in vitro pollen germination and pollen tube growth are very useful for explaining the lack of fertility (Pfahler et al., 1997).

Although there have been several studies about in vitro germination in diploid *T. pratense* (Silow, 1931; Kendall & Taylor, 1965, 1971; Kendall, 1967, 1968), studies about pollen germination in the natural tetraploid *T. pratense* were not found in the literature. Kendall & Taylor (1971), in their study on diploid *T. pratense* stated

that the rate of pollen germination increased to as high as 90%. Having explored male gametogenesis in diploid *T. pratense* Hindmarsh (1964) noted that the generative cell was divided inside the pollen tube.

The problems observed in meiotic division, differences found in the number and size of tetrad microspores, and observed variations among pollens were all reported in our previous study about the exploration of sterility causes in male gametophyte development in *T. pratense*. In this study our aim was to examine the morphological characteristics and fertility levels of *T. pratense* pollen, and the morphological characteristics of pollen tubes formed under in vitro conditions and to determine the abnormalities observed during pollen tube formation.

Materials and Methods

Anthers of the natural tetraploid *T. pratense* type E₂ were used, which was confirmed to be a plant having 28 chromosomes by counting the chromosomes at the root tips ($2n = 4x = 28$). This plant was collected from Tortum (vicinity of Erzurum, Turkey) by Elçi (1982a).

Pollen grains isolated from flower buds and flowers were immediately germinated in vitro in a culture medium (25 ml distilled water + 0.5 g agar + 6.25 g saccharose) (Elçi 1982b). When the growth of pollen tubes was suitable for the goal, having fixed some of the tubes in Carnoy (3:1) and some in Navaschin fixing solutions, they were stained with safranin-fast green, haematoxylin, aniline blue and aceto-orcein. The germination rate of the pollen grains and the growth rate of pollen tubes were determined in 1-12 h. Having counted the pollen on the slides with surface of 1 cm each, their average numbers and their germination rates with pollen tube lengths for each hour were calculated. In total, 2231, 1915, 1674, 1546, 1412, 1504, 1270 and 1268 pollen grains were examined in the 1st, 2nd, 3rd, 4th, 6th, 8th, 10th and 12th hours, respectively (Table 1). Having smeared the previously prepared culture medium on microslides, pollen germination was at 18-20 °C.

Results and Discussion

Pollen germination in tetraploid *T. pratense* continued until the end of the 12th hour and the germination rate reached 57.41% at the end of the 12th hour (Table 1). Maximum germination was noted in the 2nd and 3rd hours.

Table 1. Germinated pollen rate and pollen tube length over time.

Time	Pollen germination rate (%)	Average pollen tube length (µm)	The number of examined pollen grains
1 st hour	36.84	177.6	2231
2 nd hour	46.52	237.6	1915
3 rd hour	49.10	324	1674
4 th hour	50.45	376.8	1546
6 th hour	54.95	427.2	1412
8 th hour	55.85	475.2	1504
10 th hour	56.37	580.8	1270
12 th hour	57.41	732	1268

The germination rate reached as high as 49.10% in the 3rd hour. After the 6th hour, increases in the germination rate were small. The length of the pollen tube increased until the 12th hour, which was the last observation, time and reached an average value of 732 µm at the end of the 12th hour (Table 1).

When the structures of the pollen grains in the natural tetraploid *T. pratense* were examined, some differences in their sizes were observed (Figure 1A). The variations in pollen sizes are probably due to the tetrads that were formed at the end of microsporogenesis. While the germination rates were high among the pollen grains of normal size, no germination was observed among pollen grains of smaller size. Pollen apertures were tricolporate with reticulate ornamentation (Figure 1B). Pollen germination was mainly monosporic (Figure 1C), but as far as it has been recorded, in some pollen grains cytoplasm appeared from two or three apertures (Figure 1D). In that type of pollen, tube formation was not continual.

Silow (1931) indicated that in both compatible and incompatible types of diploid *T. pratense* pollen germinated in the stigma. However, after a quick and short period of lengthening, the growth of incompatible pollen tubes slows down and ultimately stops. He found that only a few of the living compatible pollen tubes grew and ultimately reached the ovary.

Kendall (1967), on the other hand, stated that the rate of pollen germination in diploid *T. pratense* increased to as high as 90%. According to his findings, the pollen tube normally lengthened by 2.5 mm, and when boric acid was added to the germination medium in addition to sucrose, the lengthening of the pollen tube was from 5.0

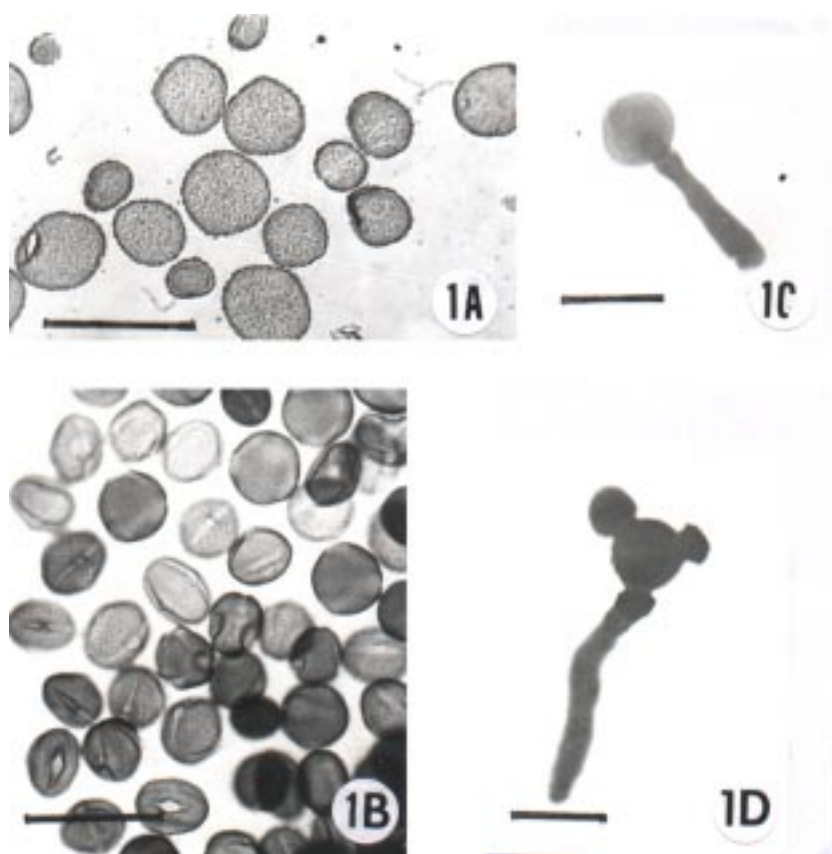


Figure 1. A) Pollen grains of *T. pratense* with various dimensions. Bar = 25 µm B) The appearance of pollen grains with tricolporate and reticulate ornamentation. Bar = 25 µm C) The growth of a monosporic pollen tube in *T. pratense*. Bar = 10 µm D) Pollen tubes from three apertures. Bar = 10 µm.

mm to 10 mm. In this research, the germination rate in tetraploid plants varied between 50 and 60%. During male gametophyte development, almost no nuclei were observed inside the pollen tube. This finding indicates that despite the germination of the pollen, the development of

male gametophyte might not occur. As far as has been noted, while some of the pollen tubes had two large nuclei (Figure 2A), some had four small nuclei (Figure 2B). Hindmarsh (1964) noted that in diploid plants, the generative cell was divided inside the pollen tube.



Figure 2. A) Two large nuclei (arrow) germinated in culture and appearing inside the pollen tube in the 2nd hour. B) Four small nuclei (arrow) inside the pollen tube. Bars = 25 µm.

Although division phases are hardly seen in tetraploid plants, the appearance of chromosomes inside the pollen tubes in some plants is accepted as evidence of division taking place inside the pollen tube. In this study, as well

as fertile ones, sterile pollen grains are also observed in in vitro medium (60-70%). In some of the germinated pollens, besides the observed bifurcation (Figure 3A) and wavy structures (Figure 3B) in the pollen tubes, some



Figure 3. Abnormalities observed in pollen growth in tetraploid *T. pratense*. A) A bifurcated monosporic pollen tube. B) A pollen tube with a wavy structure. C) An abnormally grown pollen tube due to cytoplasm deposition and wall swelling. D) An abnormal pollen tube with thickened walls. E) A pollen tube with callose formation at its tips. F) Callose plugs formed in pollen tube cytoplasm. G) A poorly grown pollen tube. Bars = 25 μ m.

swellings were also determined as well due to the cytoplasm deposition (Figure 3C). During pollen germination, some pollen grains, on the other hand, were found to be performing an abnormal germination in the first two hours. The tubes of those pollen grains in question were determined to be quite thick and the pollen cytoplasm to be stained darkly (Figure 3D). While callose (Figure 3E) at the tips of some pollen tubes and callose plugs (Figure 3F) in the cytoplasm of some others were observed, the appearance of poorly (Figure 3G) or non-grown pollen tubes were determined as several abnormalities with regard to in vitro germination of *T. pratense* pollen.

An important manifestation of incompatibility includes abnormal behaviour of the pollen tube and the heavy deposition of callose in it (Ünal, 1988). In abnormal

pollen tubes of *T. pratense* the amount of callose is greater than in normal ones. Abnormal tubes are characterized by an abnormally increased accumulation of callose in the cell wall. Furthermore, the callose plugs in these tubes are much longer and greater in number compared to those in normal pollen tubes.

In conclusion, tube growth in the majority of in vitro germinated *T. pratense* pollen grains stops due to several reasons such as callose deposition at the tips, swelling, bifurcation and bursting. In addition to the other noted barriers to fertilization in this plant (Algan & Bakar, 1996, 1997; Bakar & Algan, 1998), the stopping of tube growth due to various reasons and recorded abnormalities seem to be one of the important barriers to fertilization.

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