Turk J Bot
27 (2003) 57-61

Research Note

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

H. Nurhan BÜYÜKKARTAL
Department of Biology, Faculty of Sciences, Ankara University, Tandoğan, 06100, Ankara - TURKEY

Received: 15.05.2001
Accepted: 11.07.2002

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 monosphonic. 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 monosifoniktir. In 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 explanining 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 E_2 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 and of the 12th hour and the germination rate reached 57.41% at the and 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
1st hour	36.84	177.6	2231
2nd hour	46.52	237.6	1915
3rd hour	49.10	324	1674
4th hour	50.45	376.8	1546
6th hour	54.95	427.2	1412
8th hour	55.85	475.2	1504
10th hour	56.37	580.8	1270
12th 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 and 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 monosphonic (Figure 1C), but as far as it has been recorded, in some pollen grains cytoplasms 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

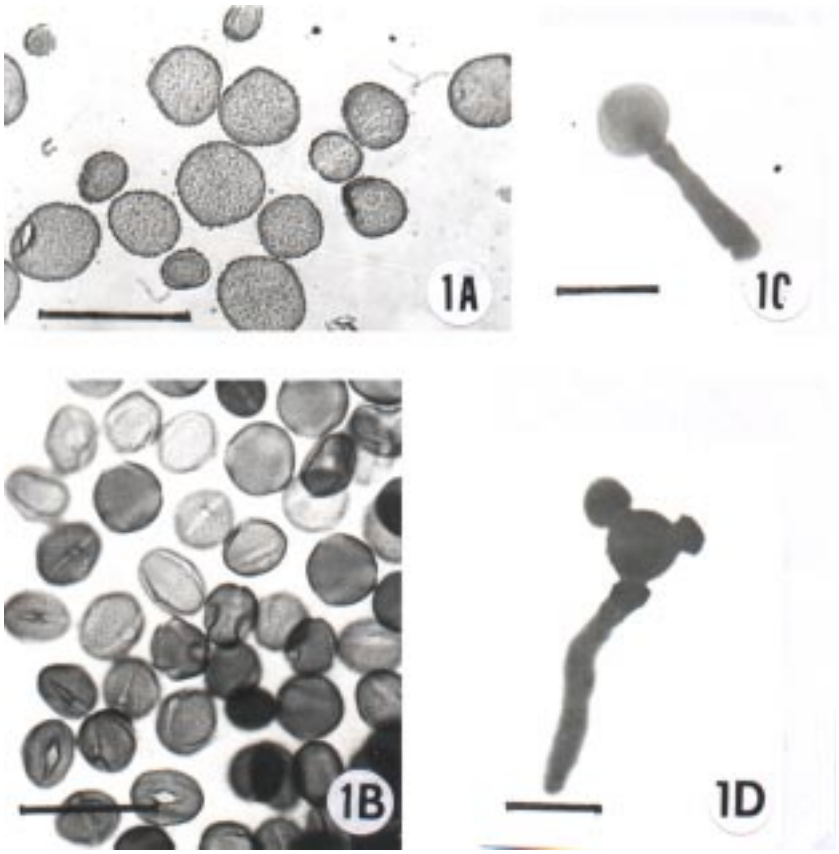

Figure 1. A) Pollen grains of *T. pratense* with various dimensions. Bar = 25 µm B) The appearance of pollen grains with tricolporate and reticulate ornamentation. Bar = 25 µm C) The growth of a monosphonic pollen tube in *T. pratense*. Bar = 10 µm D) Pollen tubes from three apertures. Bar = 10 µm.

mm to 10 mm. In this research, the germination rate in tetraplod 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.

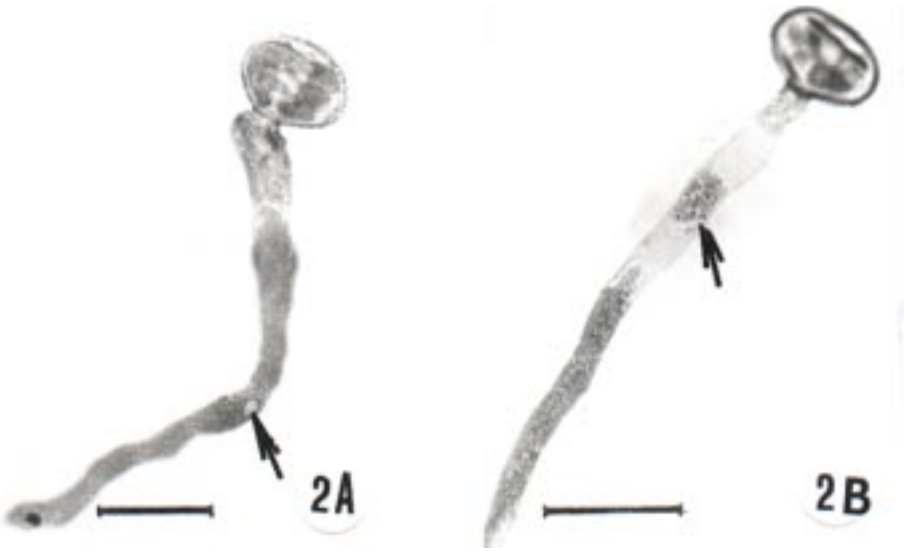

Figure 2. A) Two large nuclei (arrow) germinated in culture and appearing inside the pollen tube in the 2nd hour. B) Four small nuclei (arrow) inside the pollen tube. Bars = 25 µm.

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

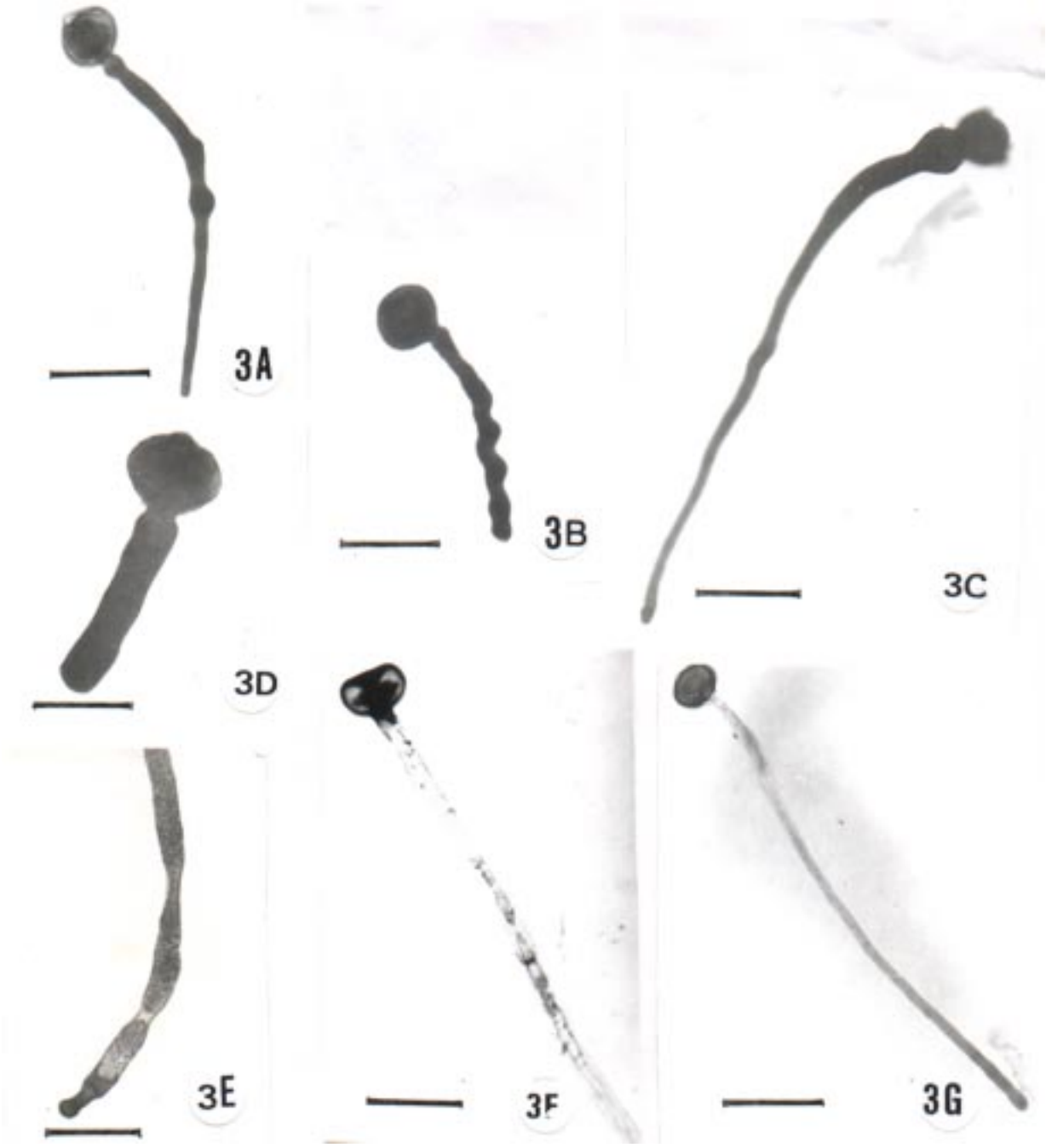

Figure 3. Abnormalities observed in pollen growth in tetraploid *T. pratense*. A) A bifurcated monosphonic pollen tube. B) A pollen tube with a wavy structure. C) An abnormally grown pollen tube due to cytoplasm deposition and wall swelling. D) An abnormal pollen tube with thickened walls. E) A pollen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

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.

References

Algan G & Bakar HN (1996). Light and electron microscopic examination of the embryo and endosperm development in the natural tetraploid *Trifolium pratense* L. *Isr J Plant Sci* 44: 273-288.

Algan G & Bakar HN (1997). The ultrastructure of the mature embryo sac in the natural tetraploid of red clover (*Trifolium pratense* L.). *Acta Soc Bot Pol* 66: 145-152

Bakar HN & Algan G (1998). Ultrastructure of the endosperm haustorium in *Trifolium pratense* L. *Acta Biol Cracov Ser Bot* 40: 15-19.

Chen CC & Gibson PB (1972). Barriers to hybridization of *Trifolium repens* with related species. *Can J Genet Cytol* 14: 381-389.

Elçi Ş (1982a). The utilization of genetic resource in fodder crop breeding. Eucarpia, Fodder crop section 13-16 September, Aberystwyth. U.K.

Elçi Ş (1982b). *Sitogenetikte gözlemler ve araştırma yöntemleri*. Elazığ: Fırat Üniv. Fen-Edebiyat Fak. Yayl. Biyoloji 3, sy. 22.

Evans AM (1962). Species hibridization in *Trifolium*. II. Investigating the prefertilization barriers to compability. *Euphytica* 11: 256-262.

Hindmarsh GJ (1964). Gametophyte development in *Trifolium pratense* L. *Aust J Bot* 12: 1-14.

Kazimierski T, Kazimierska EM & Strzyzzewska C (1972). Species crossing in the genus *Trifolium* L. *Genet Cytol* 13: 11-31.

Kendal WA & Taylor NL (1965). Growth of red clover pollen. *Crop Sci* 5: 241-243.

Kendal WA & Taylor NL (1971). Growth of *Trifolium pratense* L. Pollen tubes in compatible and incompatible styles of excised pistils. *Theor Appl Genet* 41: 275 – 278.

Kendal WA (1967). Growth of red clover pollen. II. Elongation in vitro. *Crop Sci* 7: 342-344.

Kendal WA (1968). Growth of *Trifolium pratense* L. pollen tubes in compatible and incompatible styles of excised pistils. *Theor Appl Genet* 38: 351-354.

Mackieewicz T (1965). Low seed setting in tetraploid red clover (*Trifolium pratense* L.) in the light of cytoembryological analysis. *Genet Pol* 6: 5939.

Pfahler PL, Pereira MJ & Barnett RD (1997). Genetic variation for *in vitro* sesame pollen germination and tube growth. *Theor Appl Genet* 95: 1218-1222.

Silow RA (1931). A preliminary report on pollen-tube growth in red clover (*Trifolium pratense* L.). *Welsh plant breed Stn Bull Ser H* 12: 228-233.

Taylor NL & Chen K (1988). Isolation of trisomics from crosses of diploid, triploid and tetraploid red clover. *Crop Sci* 28: 209-213.

Ünal M (1988). *Bitki (Angiosperm) Embriyolojisi*. İstanbul: Marmara Üniv. Yayın no: 495. Fen-Ed. Fak. Yayın no: 11.