Plant regeneration of natural tetraploid *Trifolium pratense* L

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ABSTRACT

The regeneration of natural tetraploid *T. pratense*, originated from Erzurum-Turkey, is reported in this study. This plant has low seed setting and hard seed problems due to polyploidy. Hypocotyl, cotyledon, apical meristems, epicotyl and young primary leaves were inoculated on MS and PC-L2 media containing different concentrations of BAP and NAA as growth regulators. The best shoot formation has been observed on explants initiated from apical meristem placed on PC-L2 medium that includes 2 mg dm⁻³ BAP and 1 mg dm⁻³ NAA. 94.4% of the shoots originated from calli were rooted on PC-L2 medium with 1 mg dm⁻³ NAA. *In vitro* organogenesis has been accomplished in the natural tetraploid *T. pratense* regenerated plants successively transferred to the field.

Key terms: red clover (*Trifolium pratense* L.), Fabaceae, organogenesis, plant regeneration.

INTRODUCTION

Trifolium, a legume, makes significant contributions to agriculture and animal feed and thus its production in the U.S. and Europe. Anatolia has been accepted as a central origin of *T. pratense* (Taylor and Smith, 1979). It is utilized as an ingredient in numerous homeopathic medicines; it has been used to relieve menopausal complaints due to its function as a phytoestrogen and also has been used in cancer treatment due to its anti-tumoral properties (Dixon, 2004).

Diploid forms of *Trifolium* species have been found suitable for agriculture (Gresshoff, 1980; Bhojwani, 1981; Grosser and Collins, 1984; Choo, 1988; Konieczny, 1995; Kaushal et al., 2006), and plant regeneration has been successfully accomplished in diploid forms of *T. pratense* L., through various methods (Phillips and Collins, 1979; Myers et al., 1989; Radionenko et al., 1994; Carillo et al., 2004).

All three varieties of *T. pratense* grown in Turkey were determined as diploid, however, *T. pratense* collected by Elci (1982) in the Tortum vicinity of Erzurum was determined as a natural tetraploid with low seed setting and hard seed problems. It has been reported that degenerations in embryo sac might affect the rate of seed setting as well as failure in fertilization (Algan and Bakar, 1997). The present work primarily aimed to develop an efficient *in vitro* regeneration system for the natural tetraploid *T. pratense*.

MATERIALS AND METHODS

Plant material and culture conditions

This study examined natural tetraploid E2 type, 2n = 4x = 28 chromosomes, *Trifolium pratense* L. (red clover) collected from the Tortum vicinity of Erzurum, Turkey, by Elci (1982). The E2-type natural tetraploid

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T. pratense L. was grown in the experimentation gardens of Ankara University's Department of Biology in the Faculty of Science. Due to contamination problems from field samples, 15-day-old aseptic seedlings with unifoliate primary leaf were used as the explant source. Seeds were first sterilized in 96% ethanol for one minute and then transferred to 10% sodium hypochlorite solution for 10 minutes (commercial sodium hypochlorite was used in the sterilization process). Then seeds were rinsed 3 times in autoclaved distilled water. After being scarified with autoclaved sandpaper, seeds were germinated on hormone-free MS medium (Murashige and Skoog, 1962). Hypocotyl (0.5-1cm), cotyledon (whole and in two fragments), apical meristem (1mm), epicotyl (0.5-1cm) and young primary leaves (whole and divided into two fragments) of aseptically grown seedlings provided explant tissues. The explants were cultured in petri dishes (100 mm x 15 mm).

MS (2% sucrose, 0.8% agar) and PC-L2 (2.5 % sucrose, 0.8 % agar, Phillips and Collins, 1979) media were used for in vitro organogenesis. Different concentrations of benzylaminopurine (BAP) and 1 mg dm⁻³ naphthalene acetic acid (NAA) (Table 1) were used in MS and PC-L2 media for shoot formation. All media were adjusted to pH 5.8 before autoclaving. Due to the darkening of calli after the third week, the shooted calli were subcultured onto the same media. All the samples were incubated at 22-24°C with a 16/8-hour photoperiod (irradiance of 42 µmol m⁻² s⁻¹ provided by cool-white fluorescent tubes).

Rooting and ex vitro acclimatization

The shoots (1-1.5 cm) obtained from MS and PC-L2 media were transferred into PC-L2 medium containing 1 mg dm⁻³ NAA for rooting in jars (100 mm x 200 mm) under aseptic conditions and incubated at 22-24°C with a 16/8-hour photoperiod (irradiance of 42 mmol m⁻² s⁻¹ provided by cool-white fluorescent tubes).

The acclimatization process of the rooted plantlets was carried out by removing the jar lids gradually with

increased durations over one week in a growth chamber. Plantlets were transplanted into sterilized garden soil. The humidity ratio of the growth chamber was gradually decreased from 80% to 50-55% throughout. When the seedlings reached a minimum leaf number of 15, they were transferred to the garden in late March and early April.

Statistical analysis

The 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). MS and PC-L2 media were compared using a paired Student's *t*-test. Each treatment was replicated three times and arranged in a completely randomized design. The data given in percentages were subjected to arcsine transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS

While apical meristem and hypocotyl explants induced callus in 3-4 days on PC-L2 medium, the same process took 5 days on MS medium. On the other hand, callus induction from the cotyledon, epicotyl, and primary leaf explants took about 1 week on MS and PC-L2 media. Great amounts of calli were grown from all the explants in 3-4 weeks. In general, different-colored white, yellow, and green calli were induced on all tested media. The yellow calli obtained on both media were more friable than the others. Among all the BAP concentrations applied, the best callus induction on MS medium was observed from apical meristem explants. Copious amount of calli were obtained from the hypocotyl and cotyledon explants on all the BAP concentrations except the medium containing 4 mg dm⁻³ BAP and 1 mg dm⁻³ NAA. The best callus induction from the epicotyl and primary leaf explants was observed on the medium containing 5 mg dm⁻³ BAP and 1 mg dm⁻³ NAA. On the PC-L2 medium supported with 2, 2.5, and 3 mg

TABLE 1

Callus response (%) of natural tetraploid *T.pratense* explants on MS and PC-L2 media. Means $\pm$ SE. Mean followed by the same letter are not significantly different, Duncan multiple comparison test within different concentrations of BAP.

Growth regulators				MS					PC-L2				
[mg dm ⁻³]				Hypocotyl	Cotyledon	Apical meristem	Epicotyl	Primary/leaf	Hypocotyl	Cotyledon	Apical meristem	Epicotyl	Primary/leaf
BAP	NAA												
2	1			84,06±0,50 ^a YG	84,28±0,28 ^a YG	84,06 ±0,50 ^a WY	74,83±0,17 ^a WYG	54,64±0,07 ^a YG	66,73±0,21 ^a YG	75,06±0,23 ^a YG	82,57±1,14 ^a WY	76,57±0,19 ^a YG	74,08±0,21 ^a YG
2.5	1			84,28±0,28 ^a YG	83,81±0,75 ^a YG	84,06±0,50 ^a WY	75,94±0,62 ^a WYG	60,24±0,24 ^b YG	75,35±0,35 ^b YG	81,44±0,24 ^b YG	84,56±0,00 ^a WY	73,36±0,11 ^b YG	84,56±0,00 ^b YG
3	1			84,06±0,50 ^a YG	83,81±0,75 ^a YG	84,03±0,53 ^a WY	67,49±0,29 ^b WYG	84,53±0,03 ^c YG	71,28±0,28 ^c YG	84,56±0,00 ^c YG	84,56±0,00 ^a WY	73,73±0,16 ^b YG	68,66±0,13 ^c YG
4	1			66,46±0,43 ^b YG	65,73±0,08 ^b YG	84,06±0,50 ^a WY	60,14±0,14 ^c WYG	56,05±0,07 ^d YG	49,28±0,26 ^d YG	50,92±0,15 ^d YG	56,42±0,18 ^b WY	55,86±0,25 ^c YG	45,09±0,09 ^d YG
5	1			84,28±0,28 ^a YG	84,31±0,25 ^a YG	84,06±0,50 ^a WY	83,78±0,78 ^d WYG	75,58±0,12 ^c YG	49,23±0,03 ^d YG	53,94±0,21 ^c YG	54,76±0,06 ^b WY	58,86±0,17 ^d YG	52,61±0,04 ^e YG

** White callus (W), Yellow callus (Y), Green callus (G)

dm⁻³ BAP and 1 mg dm⁻³ NAA, the best callus was induced from apical meristem explants. Plenty of calli were induced from all explants on all media as well as the medium containing 4 and 5 mg dm⁻³ BAP and 1 mg dm⁻³ NAA (Table 1).

Shoot formation from white and yellow calli of apical meristem started in the second and third weeks. No shoot formation was observed on the calli of hypocotyl, epicotyl, cotyledon, or primary leaf on MS medium containing different BAP concentrations. The highest shoot formation occurred from apical meristem callus on MS medium with 2.5 and 3 mg dm⁻³ BAP and 1 mg dm⁻³ NAA in 2-3 weeks. The best number of shoot per explants and the highest hyperhydric shoots resulted on MS medium with 2.5 mg dm⁻³ BAP and 1 mg dm⁻³ NAA. The highest shoot formation rate was recorded in apical meristem callus on PC-L2 medium, with 2 mg dm⁻³ BAP and 1 mg dm⁻³ NAA. Extremely low number of shoot formation was observed on the calli of hypocotyl and epicotyl on PC-L2 medium in the same concentration. Except the 2 mg dm⁻³ BAP and 1 mg dm⁻³ NAA concentration, no shoot formation was observed from cotyledon and primary leaf calli. The best number of shoot per explants (Fig. 1) and the highest

hyperhydric shoots resulted on PC-L2 medium with 2 mg dm⁻³ BAP and 1 mg dm⁻³ NAA. Some of the shoots produced from the apical meristem calli on MS and PC-L2 media established hyperhydric form (Table 2).

All the shoots induced on MS and PC-L2 media have normal trifoliate leaves. One tetrafoliate-leafed shoot formed from apical meristem callus on the MS with 4 mg dm⁻³ BAP and 1 mg dm⁻³ NAA (Fig. 2), and this shoot could not be rooted. Normal apical development occurred in some of the apical explants. Somatic embryogenesis did not occur throughout the experiments.

Figure 1: Shoots on the callus of apical meristem on PC-L2 medium (containing 2 mg dm⁻³ BAP and 1 mg dm⁻³ NAA) (Bar = 2mm).

TABLE 2

Effect of BAP on shoot induction (%) and abnormal shoot formation (%) from calli of apical meristems on MS and PC-L2 media.

Means $\pm$ SE. Mean followed by the same letter are not significantly different using Duncan multiple comparison test within different concentrations of BAP.

Growth regulators [mg dm ⁻³]		MS			PC-L2		
BAP	NAA	Shoot Formation	Number of shoots [explant ⁻¹]	Hyperhydric shoot	Shoot formation	Number of shoots [explant ⁻¹]	Hyperhydric shoot
2	1	40.81 $\pm$ 0.41 ^a	0.42 $\pm$ 0.02 ^{a b}	18.95 $\pm$ 0.51 ^a	84.56 $\pm$ 0.00 ^a	1.12 $\pm$ 0.01 ^a	38.06 $\pm$ 0.30 ^a
2.5	1	45.00 $\pm$ 0.00 ^b	0.50 $\pm$ 0.00 ^a	48.02 $\pm$ 0.15 ^b	39.23 $\pm$ 0.00 ^b	0.58 $\pm$ 0.18 ^b	31.23 $\pm$ 1.23 ^b
3	1	45.58 $\pm$ 0.58 ^b	0.38 $\pm$ 0.13 ^{a b}	33.15 $\pm$ 0.88 ^c	39.46 $\pm$ 0.70 ^b	0.61 $\pm$ 0.19 ^b	27.03 $\pm$ 0.47 ^c
4	1	32.95 $\pm$ 1.00 ^c	0.27 $\pm$ 0.02 ^b	27.13 $\pm$ 0.57 ^d	58.15 $\pm$ 1.86 ^c	0.64 $\pm$ 0.37 ^b	32.56 $\pm$ 0.62 ^b
5	1	31.33 $\pm$ 0.01 ^c	0.26 $\pm$ 0.01 ^b	32.94 $\pm$ 0.36 ^c	34.40 $\pm$ 1.87 ^d	0.22 $\pm$ 0.12 ^c	33.12 $\pm$ 0.85 ^b

Figure 2: Tetrafoliate leaf on MS supplemented with 4 mg/l BAP and 1 mg/l NAA.

The shoots were rooted at the rate of 94.4% on PC-L2 medium supplemented with only 1 mg dm⁻³ NAA. The rooting was observed in a week, and the roots continued their growth inside the medium (Fig. 3). No rooting occurred in the hyperhydric shoots when taken to rooting medium and all died.

Figure 3: The rooted shoot (young seedling) on the PC-L2 supplemented with 1 mg/l NAA.

After rooting, all the seedlings with a minimum of 15 leaves were transferred from pots to the experimentation garden by late March and early April (Fig. 4). These plants continued to grow in garden. All of them flowered in the following flowering season and produced fertile seeds.

Figure 4: The seedlings transferred to the experimentation garden.

DISCUSSION

The most widely used forms of *T. pratense* are 2n = 14 diploid types in tissue culture research and in various studies. This study is the first tissue culture study carried out with the natural tetraploid *T. pratense* L. 2n = 4x = 28 grown in Turkey. Researchers used *T. pratense* aseptic seedlings of different ages as explant sources (Phillips and Collins, 1979; Bhojwani, 1981; Myers et al., 1989). In almost all the studies, apical tip and hypocotyl explants have been preferred and also were utilized in our study. However, the ages of the seedlings used by the researchers display variations. According to our results, 15-day-old seedlings are the most suitable explant sources due to their totipotency properties.

Apical meristem and hypocotyl explants induced calli in a shorter time on PC-L2 medium compared to MS medium. On the other hand, MS medium responded better in general regarding callus formation compared to PC-L2 medium. The medium was not found to have an impact on callus color; morphologies of the calli on both medium were similar. While white and

yellow calli were induced from the apical meristems in both media, the calli from the other explants were mainly yellow and green. Myers et al. (1989) obtained friable calli from *T. pratense* on L2 (Phillips and Collins, 1979). In our study, the yellow calli obtained from all of the explants were found to be friable calli.

Phillips and Collins (1979) obtained high numbers of shoots in 2-3 months from the meristem callus placed in a medium containing 0.006 picloram and 0.1-10 mg dm⁻³ BAP concentrations, whereas, in our study, apical meristem callus produced shoots in a shorter time (2-3 weeks). PC-L2 medium responded better in terms of shoot formation compared to MS medium. Shoot number per explant was higher on the PC-L2 medium supplemented with 2 mg dm⁻³ BAP and 1 mg dm⁻³ NAA compared to MS medium. Moreover, higher numbers of hyperhydric shoots were observed in the MS medium supplemented with 5 mg dm⁻³ BAP and 1 mg dm⁻³ NAA in comparison to the PC-L2 medium. The results of our experiments revealed PC-L2 to be the best medium for plant regeneration of the natural tetraploid *T. pratense*. The best plant growth regulator combination was found to be 2 mg dm⁻³ BAP and 1 mg dm⁻³ NAA.

Phillips and Collins (1979) achieved 85% rooting on hormone-free MS medium in 2-4 weeks. Researchers have rooted the shoots on different media (Gresshoff, 1980; Bhojwani, 1981; Myers et al., 1989). In terms of rooting, the same rooting medium has yielded optimum results for shoots obtained from both media in our study. The observation of rooting at the end of the first week and a rooting rate of 94.4 % are noteworthy achievements. No rooting was done on MS medium after the achievement of such a high rate on PC-L2 medium.

Several researchers, after adjusting the rooted shoots to the air medium, have transferred them into different mixtures of soil and applied different rates of humidity (Phillips and Collins, 1979; Bhojwani, 1981). In our study, no specific soil mixture has been used. The soil used was sterilized, normal garden soil, and the temperature was set to 22-24°C with an initial humidity

rate of 80%. Researchers have mentioned that they transferred the regenerated plants to a greenhouse. After transferring the plantlets into individual pots containing soil and keeping them in the greenhouse briefly, we completely transferred them to external medium by transferring them to our experimentation garden. The immediate adaptation of the natural tetraploid *T. pratense* to garden soil can be explained by the superior adaptation ability of ploidy-containing plants to difficult circumstances. Our plants continued growing (100%) in soil. All of them flowered in the following flowering season and produced fertile seeds.

This study has given a method for rapid propagation of natural tetraploid *T. pratense* *in vitro*. As previously mentioned, these diploid plants contain components functioning as phytoestrogens. Thus, the plant regeneration described in this study may open the possibility for using biotechnological techniques to obtain high production of naturally occurring secondary metabolites (flavonoids) in natural tetraploid *T. pratense* L.

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