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BRIEF COMMUNICATION

In vitro regeneration of *Trifolium glomeratum*

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Abstract

In vitro regeneration of *Trifolium glomeratum*, a leguminous forage species, was attempted through leaf, petiole, cotyledon, hypocotyl, collar and root explants and two media combinations. Root and collar explants showed no callus induction. Medium with 0.05 mg dm^{-3} α-naphthaleneacetic acid (NAA) and 0.10 mg dm^{-3} N^6-benzyladenine (BA) was more effective for hypocotyl explant whereas cotyledon and petiole explant were more responsive to 5.0 mg dm^{-3} NAA and 1.0 mg dm^{-3} BA. Friable, green calli obtained from petiole explant on this medium showed organogenetic potential. Modified root-inducing medium having 0.21 mg dm^{-3} indole-3-acetic acid and 2.5 % sucrose was successful for root induction and plantlets were successfully transferred to field after hardening and *Rhizobium* inoculation.

Additional key words: auxins, clovers, cytokinins, tissue culture.

The genus *Trifolium*, comprises of about 300 species of which twenty-five are important as cultivated forages and pasture crops for both wild and domestic animals. *T. glomeratum*, an annual diploid species (2n=2x=16) with good forage quality, is frequently inhabited in dry places of Europe. The species possesses close affinity with agriculturally important species (*e.g. T. repens*, *T. alexandrinum*) (Malaviya *et al*. 2005), and hence, may be utilized as donor for desirable traits such as resistance to root rot and stem rot (Bhaskar *et al*. 2002). Efficient *in vitro* regeneration protocol is a prerequisite to conduct biotechnological experiments (Espino *et al*. 2004, Zapata *et al*. 2004). Hence, the present investigation was carried out to study the effect of different media (growth regulator combinations) and explant towards *in vitro* response and to develop a suitable *in vitro* regeneration protocol.

Healthy seeds of *Trifolium glomeratum* L. (accession EC 401700) were surface sterilized using 0.1 % $HgCl_2$ for 1 min followed by two to three washings in distilled sterilized water. Seeds were germinated on MS_0 medium (Murashige and Skoog 1962), devoid of any growth regulators, containing 3 % sucrose and agar (0.7 %). For callus induction, shoot induction and somatic embryogenesis, L2 basal composition as suggested by Phillips and Collins (1984) was used. Callus induction was attempted on 'A' medium [having 0.05 mg dm^{-3} α-naphthaleneacetic acid (NAA), and 0.10 mg dm^{-3} N^6-benzyladenine (BA)] as well as 'D' medium (having 5.0 mg dm^{-3} NAA and 1.0 mg dm^{-3} BA). In shoot inducing medium 'E', NAA and BA were 0.0008 mg dm^{-3} and 0.15 mg dm^{-3} respectively. For somatic embryogenesis, 2,4-dichlorophenoxyacetic acid (2,4-D; 0.001 mg dm^{-3}) and adenine (3.225 mg dm^{-3}) were used in somatic embryogenesis inducing medium (SEIM) medium. Root induction was done on RL1 medium consisting of RL basal medium (Phillips and Collins 1984) and 0.21 mg dm^{-3} indole-3-acetic acid (IAA) and 2.5 % sucrose. Six explants *viz.*, leaf, petiole, hypocotyl, cotyledon, collar and root were taken from 20 to 30-d-old healthy seedlings and inoculated in callus inducing media. The cultures were maintained at 24 ± 2 °C. Initially the explants were kept in dark for callus induction but 5 d after inoculation the cultures were grown under 8-h photoperiod with irradiance of 63 μmol m^{-2} s^{-1} (phosynthetically active radiation). After shoot emergence, the photoperiod was increased to 10 - 12 h for shoot multiplication and further growth. The experiment was carried out in 5 sets. Each set had 3 culture tubes with 2 explants in each tube. A total of 30 explants were cultured per explant media combination.

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Abbreviations: BA - N^6-benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid, IAA - indole-3-acetic acid; NAA - α-naphthaleneacetic acid.

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The observations were recorded on 1-month-old cultures for nature, colour and growth rate of the callus. Calli growth less than 5 mm in diameter were recorded as slow. After 30 - 35 d, the calli were split and sub-cultured in 10 - 20 tubes to either shoot inducing media 'E' or somatic embryogenesis inducing medium (SEIM). Periodic observations were taken and in case of shoot induction, the shoots were multiplied by one more round of sub-culturing in the same culture medium. The shoots were then split and sub-cultured on root induction medium. The lower part of the tubes was covered with black paper to facilitate the root induction. Repeated sub-culturing of shoots was done in cases where no root induction was observed.

Complete plantlets with root and shoot were hardened by keeping the plants out of culture room at room temperature. For *Rhizobium* inoculation, nodules collected from the old plants from the field were macerated in distilled water and the root of regenerated plants was dipped for overnight following Roy *et al*. (2004). The plants were then transferred to pots having soil and sand in equal proportion. The process helped in induction of nodules in the regenerated plants in 6 - 7 d.

The data obtained for callus induction in different media-explant combinations was grouped and mean ± standard deviation was calculated. Analysis of variance (2-way *ANOVA*) test was carried out to analyze the effect of interaction of various factors (Sokal and Rohlf 1969). Duncan's multiple range test was used to compare the significance of the differences, if any, among the mean of different experimental sets (Duncan 1955, Harter 1960).

Differential response of media-explant interaction was observed as indicated by parameters such as callus induction frequency, nature and growth rate of callus and sub-culturing response towards organogenesis and embryogenesis. Leaf explant exhibited poor response (6.7 and 3.3 %) in both the callus inducing media 'A' and 'D' and in most cases necrosis was observed within 8 - 10 d (Table 1). Very poor response (4.0 %) of petiole for callusing was observed in 'A' medium even after 25 - 30 d of culture and the explants showed necrosis. However, in 'D' medium, swelling in 73.3 % petiole explant was induced within 10 - 11 d and small, green, friable callus was formed in about 18 - 20 d. Callus growth was better in this medium and it developed to 6 - 6.5 mm in diameter after nearly six weeks. Cotyledons in 'A' medium, showed slight swelling in 3.3 % cultures only, while rest showed browning of the tissues within 10 d. However, cotyledon explant in 'D' medium enlarged and thickened in 7 - 8 d and small yellow callus was formed in 12 d on the periphery of the explant which remained yellow, compact and slow growing. Hypocotyl explants showed poor response in 'D' medium, however, in 'A' medium, explants responded positively and swelling was induced in 10 - 11 d resulting in small compact, yellow callus formed in 20 - 24 d. The callus was slow growing and developed to 4.5 - 5 mm diameter in 5 weeks. Collar and root explants did not show any response for callus induction in any media. The Duncan's multiple range test indicated that cotyledon + 'D' medium combination was significantly better ($P < 0.01$) than other combinations for callus induction. It was followed by hypocotyl + 'A' medium and petiole + 'D' medium combinations.

The calli obtained from different explants were split and sub-cultured in 'E' medium. Calli derived from hypocotyl, leaf and cotyledon showed slight proliferation in 20, 10 and 30 % cultures, respectively, but no organogenesis was observed even after repeated sub-culturing. Browning of the callus started within 11 - 12 d. The callus developed from petiole on 'D' medium showed emergence of small shoot like structure within 15 d of sub-culturing in 'E' medium. The multiple shoot formation was observed in these tubes in next 10 - 12 d. These shoots were then split in many tubes and again sub-cultured on 'E' medium for shoot multiplication. After a total of 35 - 40 d in 'E' medium the shoots were split and a total of 57 shoots were cultured on root inducing medium 'RL1'. Root initiation was first noticed within 7 - 9 d followed by emergence of multiple roots and secondary roots in next 10 d. Thus, complete plantlets (with root and shoot) were obtained in 35 tubes (61.4 %) in about 14 weeks (Fig. 1) while rest of the shoots either did not show any response or only shoot growth was observed. The plants were taken out, freed of agar and kept for an additional day with the roots submerged in sterilized distilled water containing crushed nodules from *T. alexandrinum* plants growing in field and

Table 1. Callusing response of *T. glomeratum* explants in different media. Means with the same letters were not significantly different at $P < 0.01$ (Duncan 1955).

Explant source	NAA [mg dm^{-3}]	BA [mg dm^{-3}]	Number of explants	Callus induction [%]	Callus
Leaf	0.05	0.1	30	6.7 ± 9.13^{a}	compact, yellow
	5.00	1.0	30	3.3 ± 7.45^{a}	compact, yellow
Petiole	0.05	0.1	27	4.0 ± 8.94^{a}	friable, yellow
	5.00	1.0	27	73.3 ± 19.00^{b}	friable, green
Cotyledon	0.05	0.1	30	3.3 ± 7.45^{a}	friable, hyaline
	5.00	1.0	30	93.3 ± 14.91^{c}	compact, nodular, yellow
Hypocotyl	0.05	0.1	30	66.7 ± 11.79^{b}	compact, yellow
	5.00	1.0	30	3.3 ± 7.45^{a}	friable, hyaline

covered with cellophane paper to maintain high humidity. These plants were protected from direct sunlight for the first 3 - 5 d in the field. Out of 35 plantlets transferred to field, 12 (34.29 %) plants could be established while other did not survive after 5 to 6 d.

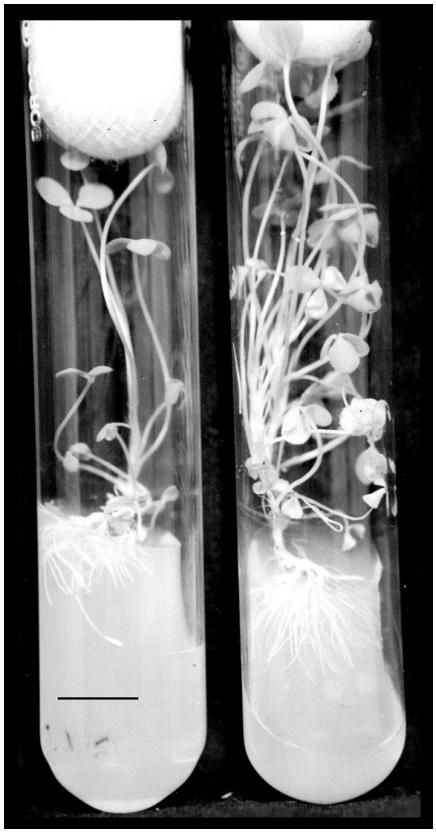

Fig 1. *In vitro* regeneration in *Trifolium glomeratum* (*bar* = 1 cm).

In parallel to above experiment, the calli from hypocotyl, petiole and cotyledon explants were sub-cultured in SEIM medium but the calli from cotyledon and petiole did not show any sign of embryoid formation. Only callus proliferation was seen which turned brown after 20 - 25 d. Two out of 20 cultures with calli obtained from hypocotyl on 'A' medium showed some embryoid like structures (Fig. 2). However, these could not be further used, as they turned brown after 20 d.

The callus induction frequency, nature of calli and their growth rate was found to be dependent on media-explant combinations. Medium 'D' with high concentrations of growth regulators was found to be superior to medium 'A' with low concentration of growth regulators for callus induction when all the observations were summarized. The response was especially high in 'D' medium for petiole and cotyledon explants. The differential response of explants of different species to varying hormonal concentration can be attributed to the nature of explant tissue, its genetic potential for regeneration and its nutritional requirement. It has been observed that nutritional requirements for optimal growth of a tissue *in vitro* vary and even tissues from different parts of a plant may have different requirements for satisfactory growth (Espino *et al*. 2004, Zhang *et al*. 2004).

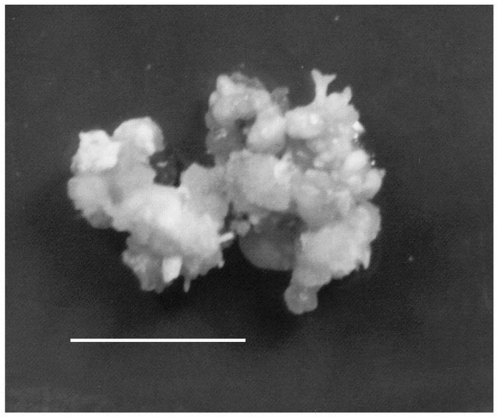

Fig 2. *In vitro* somatic embryogenesis in *Trifolium glomeratum* (*bar* = 1 cm).

Skoog and Miller (1957) demonstrated in tobacco that high ratio of auxin to cytokinin favoured root formation, the reverse favoured shoot formation and an intermediate ratio promoted callus proliferation. In our study, ratio of auxin and cytokinin were tried in two combinations for callus induction, and regeneration. The results established media-explant interaction effect in addition to ratio of auxin to cytokinin.

It was observed that only friable, green calli were responsive to organogenesis in shoot inducing medium 'E'. Successful shoot induction was observed only in calli derived from petiole in 'D' medium. The modified root inducing medium 'RL1' (after Phillips and Collins 1984) was reported to be good in *T. alexandrinum* (Tiwari 2001). In this species, we tried only this medium and found it to be responsive. Roots were induced in almost half of the shoots cultured.

Thus, in this genotype petiole in 'D' medium was found to be the best combination for morphogenetic callus induction. The study also resulted in development of protocol for regeneration of plants in this species.

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