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CALLUS AND SHOOT-TIP CULTURE OF EIGHT *TRIFOLIUM* SPECIES IN VITRO WITH REGENERATION VIA SOMATIC EMBRYOGENESIS OF *T. RUBENS*

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SUMMARY

Systems in vitro developed for *Trifolium pratense* were evaluated for use with seven other important Trifolium species. Calli were successfully initiated from seedling explants of *T. alpestre*, *T. campestre*, *T. incarnatum*, *T. medium*, *T. pratense*, *T. repens*, *T. rubens* and *T. subterraneum*.

Suspension cultures of all species except *T. campestre* and *T subterraneum* were initiated from callus and colonies were successfully recovered from replates of all species examined. Successful plant regeneration was obtained from both callus and suspension-derived cultures of *T. rubens* by somatic embryogenesis.

Shoots from all species except *T. campestre* were clonally propagated using meristem-tip culture methods. These shoots were successfully rooted, except for those of *T. incarnatum*. These results indicate that the manipulation in vitro of several Trifolium species is possible with available systems.

INTRODUCTION

Methods for culturing species in the genus Trifolium in vitro have been developed in recent years. Callus cultures of *T. hybridum* L. (alsike clover) [1] and *T. subterraneum* L. (subterranean clover) [2] have been reported. Additionally, callus cultures have been established and plants regenerated from *T. repens* L. (white clover) [3—5], *T. incarnatum* L. (crimson clover) [6], *T. alexandrinum* L. (berseem clover) [7] and *T. pratense* L. (red clover) [6,8]. Plants have also been obtained from meristem-tip cultures of *T. pratense* [9] and *T. repens* [10]. Cell suspension cultures of *T. subterraneum* have been established [2] and plants have been regenerated from cell suspen-

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sion cultures of *T. pratense* [11] and *T. alexandrinum* [7]. In addition, plants were regenerated from protoplast cultures of *T. repens* [3]. Haploid plants of *T. alexandrinum* [7] have been reported from anther cultures.

Most recently, Phillips et al. [12] adapted their L2-basal medium for use with embryo rescue techniques. This medium was used to successfully obtain a hybrid between *T. pratense* and *T. sarosiense*, a perennial subspecies of *T. medium*.

Gresshoff [3] has pointed out similarities among various media used for the culture of different clovers and noted that there is some plasticity in their cultural requirements. In this study, the tissue culture methods developed for *T. pratense* by Phillips and Collins [8,9,11,12] were evaluated for seven additional clover species which are of economic importance or could potentially be hybridized with *T. pratense*. The species evaluated were *T. alpestre* L., *T. campestre* Schreb. (large hop clover), *T. incarnatum*, *T. medium* L. (zigzag clover), *T. repens*, *T. rubens* L. and *T. subterraneum*. *T. pratense* (cv. Kenland) was cultured for comparison with the other species.

MATERIALS AND METHODS

Seeds of each species used were sacrificed, surface sterilized, and germinated following the procedures outlined by Collins and Phillips [13].

Ten seedlings were selected from each species when their length averaged 4 cm. Only six seedlings were available for *T. incarnatum* and *T. medium*. The shoot-tip of each selected seedling was excised and placed on ML8 medium as described by Phillips and Collins [9]. The remaining portions of the seedlings were sectioned and inoculated into culture on L2 medium [8] and grown under continuous cool white fluorescent lights supplemented with incandescent bulbs (average intensity was 550 $\mu Em^{-2} s^{-1}$) for 1 month.

The peripheral callus formed on each seedling section was subcultured onto fresh L2 medium. The shoot-tips were also transferred onto fresh ML8 medium. At the same time, calli of *T. alpestre*, *T. medium*, *T. repens* and *T. rubens* were used to initiate cell suspension cultures. Cell suspensions of the other species were not started at this time due to an insufficient supply of callus, although cell suspensions of *T. incarnatum* and *T. pratense* were successfully initiated at a later date. The cell suspensions were initiated and cultured as described by Phillips and Collins [11] using SL2 medium.

The cell suspensions were subcultured weekly by pouring 10—15 ml of the suspensions into 30—40 ml of fresh medium. Following the fifth and subsequent subcultures, 3—5 ml of each suspension were plated onto both L2 and LSE agar-solidified media [9] immediately preceding the routine subculture of the cell suspensions.

All the calli growing on L2 medium were transferred after an additional month onto LSE medium. *T. campestre* was not included in the transfer since the callus had failed to grow. These cultures were subcultured onto fresh LSE medium at monthly intervals for three months.

Well-formed shoots which developed from the shoot-tip explants on ML8 medium were separated and placed on RL medium. Insufficiently developed shoots were subcultured again onto ML8 medium. Shoots of *T. medium* were subcultured onto ML11 medium (ML8 medium containing only 0.001 mg/l picloram*) to minimize callus growth and promote shoot development.

The cultures maintained on RL (previously called RPC) medium [9] were transferred onto fresh medium at monthly intervals until roots were visible and well developed. Rooted plants were potted, hardened, and transferred to the greenhouse [8].

The shoots obtained from somatic callus tissues were potted following root initiation on RL medium. Plants which initially regenerated both shoots and roots were potted directly.

RESULTS AND DISCUSSION

The results of the responses in vitro of the species tested are summarized in Table I. The only two species which did not respond well on L2 medium were *T. campestre* and *T. subterraneum.* Both of these species are annual rather than perennial species. Subcultured *T. campestre* and *T. subterraneum* callus turned brown quickly and ceased growing, suggesting that some component(s) in the medium was inhibitory. In its present form, the basal medium is unsuitable for the efficient culture of these species. Although *T. medium* callus showed the greatest volume increase, its weight increase was not as great as that of *T. rubens*, suggesting that callus of the former had a lower density.

Callus recovery from suspension cultures

Suspension cultured cells of all species evaluated with the exception of *T. medium* failed to develop into a callus when replated onto LSE agar-solidified medium. Very small, slow-growing colonies of *T. medium* callus developed. The LSE medium was considered an unsatisfactory callus recovery medium for these Trifolium species.

In contrast, suspension cultured cells replated on L2 medium grew much better. The plate-outs of both *T. alpestre* and *T. repens* produced callus which was considerably more vigorous than that which had remained on solidified L2 medium. This result suggests that selection for growth under these tissue culture conditions took place during culture as cell suspensions.

Plant regeneration

Plants were regenerated from some of the small aggregates of *T. rubens* cells recovered from suspension cultures as callus on L2 medium. These plants developed from well-formed somatic embryos which germinated and

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TABLE I

COMPARATIVE RESPONSE IN VITRO OF EIGHT TRIFOLIUM SPECIES ON VARIOUS MODIFICATIONS OF THE L2 BASAL MEDIUM

0, death; 1, no response; 2, poor response; 3, good response; 4, very good response; 5, excellent response; blank, not available.

	T. alpestre	*T. campestre*	*T. incarnatum*	*T. medium*	*T. pratense*	*T. repens*	*T. rubens*	*T. subterraneum*
Explant to callus on L2	2	1	3	5	3	2	4	2
Callus growth on L2	2	0	3	5	3	2	4	1
Suspension inoculation and growth on SL2	3		3	5	3	2	4	
Regeneration on LSE	1		1	1	1	1	3	
Rooting potential on RL[a]	3					3		
Shoot meristem clonal propagation on ML8	3		3	3	2	3	3	4
Callus growth on LSE	2		2	2	2	2	3	0
Recovery efficiency of cells as callus on L2	3		3	5	3	2	4	
Recovery efficiency of cells as callus on LSE	1		1	2	1	1	2	

[a] For meristem-derived plants.

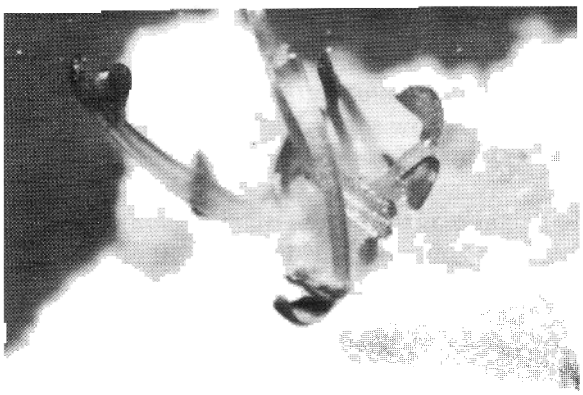

Fig. 1. Embryogenic callus and plantlets of *T. rubens* following replating of suspension cultured cells on L2 medium.

produced an initial unifoliate leaf followed by the production of trifoliate leaves (Fig. 1).

Differentiation of suspension derived *T. rubens* calli into somatic embryos was further enhanced by transferring the regenerating calli to LSE medium. Previously non-morphogenic callus of *T. rubens* also regenerated plants after this transfer.

The calli which had not been used to initiate cell suspension cultures were placed on LSE medium. Callus from one of the ten *T. rubens* genotypes initiated somatic embryogenesis three weeks after the initial transfer to LSE medium. By the fourth subculture onto LSE medium, five of the genotypes had produced somatic embryos. *T. rubens* is very amenable to tissue and cell culture manipulation. Of the *T. rubens* genotypes evaluated, 50% were capable of regenerating plants.

Shoot-tip culture

Shoot-tips were successfully proliferated for all the species evaluated with the exception of *T. campestre*, *T. medium* shoot-tips produced fewer shoots and more callus than the other species. This problem was corrected by using ML11 medium which contains only one third the level of picloram compared to ML8 medium.

CONCLUSION

Shoot-tip culture of *T. alpestre*, *T. incarnatum*, *T. medium*, *T. repens*, *T. rubens* and *T. subterraneum*, using the method originally developed for *T. pratense*, offers an efficient system for clonal propagation of hybrids obtained between these species and *T. pratense*. Shoot meristem-tip culture could also be used to obtain virus-free plants and propagate desirable breeding lines of these species.

Efficient cell suspension cultures were obtained for *T. alpestre*, *T. incarnatum*, *T. medium*, *T. repens* and *T. rubens*. Plants were regenerated by somatic embryogenesis in *T. rubens*. Since callus cultures of *T. alpestre*, *T. incarnatum*, *T. medium* and *T. repens* grow successfully, efficient regeneration of plants should be possible by modifying the composition of the media used.

Overall, *T. pratense* performed significantly better than *T. campestre*, *T. incarnatum* and *T. repens*. This is not unexpected since the L2 medium and its modifications were designed specifically for a broad range of *T. pratense* genotypes. Both *T. medium* and *T. rubens* performed better than *T. pratense*. These two species appear to be closely related to *T. pratense* in nutritional requirements. In addition to the vigorous growth of *T. rubens* in culture, its high regeneration frequency may make it an ideal species to use as a model system for culture and manipulation of Trifolium species in vitro and perhaps for other legumes as well.

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