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# Genotypic Variation in Plant Regeneration from Calli and Protoplasts of Alfalfa, *Medicago sativa* L.

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Four cultivars of alfalfa, *Medicago sativa* L., were tested in vitro for their ability to produce adventitious embryos followed by whole plants in calli derived from hypocotyl. Four kinds of media were used to survey the response of callus induction, callus culture for pre-embryogenesis, adventitious embryo formation and germination. The variation of response in plant regeneration was observed between cultivars and also among the individual plants of each cultivar. Adventitious embryo formation was rapidly reduced after 5 to 7 subcultures in calli derived from the hypocotyl. A system was established for achieving plant regeneration from hypocotyl-derived callus protoplasts. About 2 to 3-week-old calli after transplantation onto the new medium were incubated in an enzyme mixture containing 4% Cellulase Onozuka RS, 1% Macerozyme R-10, 0.2% Pectolyase Y-23 and 0.7 M mannitol for 1 hr to liberate protoplasts. Modified 8p medium of Kao and Michayluk[1] supported cell division and colony formation. The calli and whole plants were obtained by the same kinds of media as used for callus induction from hypocotyl and plant regeneration.

The application of tissue and cell culture and genetic manipulation to crop improvement is largely dependent upon the ability of plant regeneration from cultured calli, cells and protoplasts. The ability of plant regeneration from cultured cells and tissues has been clarified to depend upon the genotype in many species including legumes such as *Trifolium*,[2] *Cajanus*,[3] *Coronilla*[4] and *Lotus*.[5] In alfalfa, *Medicago sativa* L., the induction of somatic embryogenesis on calli varies between cultivars and also among the individual plants of a cultivar.[6-8] The wide range of genotypic variation in the somatic embryogenesis and plant regeneration may sometimes be a hindrance of the work of in vitro technique for crop improvement.

The present work was initiated to isolate the plants with genotype of high plant regeneration from calli and protoplasts. They could be useful in genetic manipulation studies.

## Materials and Methods

*Plant materials, callus induction and culture.* Cultivars of alfalfa used in this study are DuPuit, Rangelander, Rambler and Vernal. The seeds of each cultivar were immersed in 75% ethanol for several seconds, and then washed several times with sterilized distilled water to remove the ethanol completely. Subsequently, the seeds were immersed in a 1.5% solution of sodium hypochlorite for 15 min and then thoroughly washed several times with sterilized distilled water. The seeds were germinated on the medium of Nitsch and Nitsch[9] without growth regulators. Four kinds of media used for callus induction and plant regeneration are the same as those used by Brown and Atanassov.[7] About 1 cm of segments of hypocotyls of 2-week-old plantlets were cultured on

the B 5 medium[10] modified to contain 3,000 mg/*l* $KNO_3$, 895 mg/*l* $CaCl_2 \cdot 2H_2O$, 500 mg/*l* $MgSO_4 \cdot 7H_2O$, 800 mg/*l* L-glutamine, 100 mg/*l* serine, 10 mg/*l* glutathione, 1.0 mg/*l* adenine, 30 g/*l* sucrose, 1.0 mg/*l* 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/*l* kinetin. After 1 month of the cultures, calli were formed. They were transplanted onto Schenk and Hildebrandt medium[11] containing 11 mg/*l* 2,4-D and 1 mg/*l* kinetin. After 3 weeks the calli were transferred to Blaydes medium[12] modified to contain 100 mg/*l* myo-inositol and 2 g/*l* yeast extract and, 2 to 4 weeks after, adventitious embryos were formed, and they were transplanted to B 5 medium without growth regulators for plantlet formation. All cultures were maintained under light conditions at 25℃.

*Protoplast isolation and culture.* The calli induced from hypocotyls were subcultured on the medium for callus induction at intervals of 1 month. Two to 3-week-old calli after transplantation onto the fresh media were used for the isolation of protoplasts. A solution containing 4% Cellulase Onozuka RS, 1% Macerozyme R-10, 0.2% Pectolyase Y-23 and 0.7 M mannitol (pH 5.8) was used for the protoplast isolation. The enzyme-callus mixture was incubated at 28℃ for 1 hr in a shaker bath (60 shakes/min). The protoplasts were separated from undigested cell clumps by 8 layers of gauze. The enzyme was removed by 4 successive washings with 0.7 M mannitol (pH 5.8) by centrifugation at 80 $\times g$ for 4 min each. The isolated protoplasts at a density of $1.5 \times 10^5$/ml were cultured in thin layers of a modified 8 p liquid medium[1] containing 0.5 mg/*l* BA instead of zeatin and lacking coconut water. The culture dishes were kept under dark conditions at 25℃. After initiation of cell division a fresh modified 8 p medium was added and then the culture dishes were moved under light conditions at 25℃. A fresh medium was added 2 or 3 times at intervals of 10 days. After about 2 months of culture, colonies were formed. The procedures for callus formation from the colonies, adventitious embryo induction and plantlet formation are the same as in the case of hypocotyl callus culture.

## Results and Discussion

*Plant regeneration from callus culture*

All hypocotyl-segments of different individuals in all cultivars produced calli on the modified B 5 medium for about 1 month. Then, embryogenesis in the calli occurred on a modified one of Blaydes medium[12] after callus culture for pre-embryogenesis on a medium with high concentration of 2,4-D (**Fig. 1-A**). In a primary culture, the response of embryogenesis in the calli derived from hypocotyl was strongly dependent upon genotypes of cultivars and also individual plants (**Table 1**). The cultivars, Rangelander and Rambler, formed the adventitious embryos with very high frequencies in the individuals of these varieties, while the cultivars, DuPuit and Vernal, formed those with low frequencies. Bingham et al.[6] and Brown and Atanassov[7] found similar variations in cultivars of alfalfa used for regeneration in calli derived from hypocotyl. Kao and Michayluk[8] have also reported the genotypic variation in the embryogenesis in calli derived from leaf mesophyll protoplasts and cell suspensions derived from isolated shoot tips. Genotypic variation in the somatic embryogenesis would appear to be a widespread phenomenon in alfalfa. This may be due to the fact that the alfalfa is an open-pollinated species with a great deal of inter- and intravarietal genotypic variation.

**Table 1.** Formation of adventitious embryos on the calli derived from different individuals of four cultivers in a primary culture.

| Cultivar | No. of callus derived from different individuals[a] | No. of callus formed adventitious embryo | % of callus formed adventitious embryo |
|---|---|---|---|
| DuPuit | 16 | 2 | 12.5 |
| Rangelander | 17 | 12 | 70.5 |
| Rambler | 7 | 4 | 57.1 |
| Vernal | 17 | 1 | 5.8 |

[a] One callus was derived from each plant.

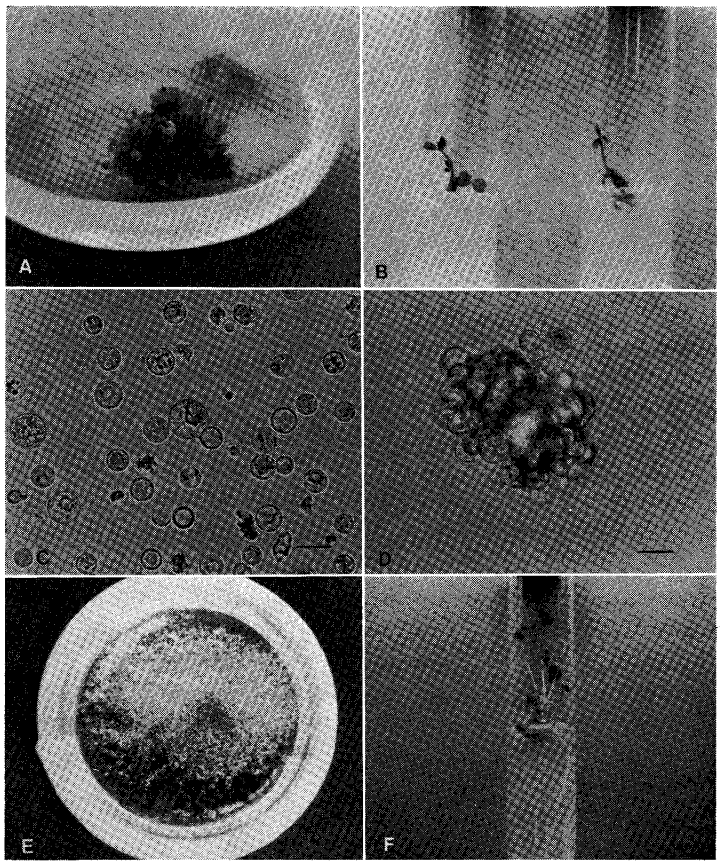


**Fig. 1.** A: Adventitious embryogenesis in a hypocotyl-derived callus on the modified one of Blaydes medium. B: Germination of the adventitious embryos on the B 5 medium without growth regulators. C: Isolated protoplasts from the callus. D: A cell cluster formed in the modified 8 p medium. E: Colony formation. F: A regenerated plantlet by the protoplast culture. Bars represent 20 $\mu$m.

**Table 2.** The loss of formation ability of adventitious embryos on the calli after 5-7 subcultures.

| Cultivar | No. of callus formed adventitious embryo in a primary culture | No. of callus formed adventitious embryo after 5-7 subcultures | % of callus formed adventitious embryo |
|---|---|---|---|
| DuPuit | 2 | 0 | 0 |
| Rangelander | 12 | 1 | 8.3 |
| Rambler | 4 | 1 | 25.0 |
| Vernal | 1 | 0 | 0 |

The adventitious embryo removed from the calli germinated (**Fig. 1-B**) and developed into complete plantlets on the B 5 medium[10] without growth regulators. The frequency of embryogenesis, however, was rapidly reduced after 5 to 7 subcultures (**Table 2**).

*Plant regeneration from protoplast culture*

Calli at less than three weeks after transplantation onto the fresh medium were found to remain

in the state which was the most responsive to protoplast isolation (**Fig. 1-C**) and cell division. This is due to the fact that the conditions of donor callus may affect the yield of isolated protoplasts as well as their subsequent metabolic activity and cell division efficiency.[13] When the protoplasts were cultured in a modified one of 8 p medium,[1] they divided within 4 days after the isolation. A peak in cell division activity was observed between 1 to 2 weeks (**Fig. 1-D**). It was necessary to add a fresh medium 2 or 3 times at intervals of 10 days to obtain colonies (**Fig. 1-E**). The small, less vacuolated protoplasts which may be derived from parts of meristematic tissue of callus appeared to divide at the highest frequency and small cell colonies could be formed in 2 months after protoplast isolation. Protoplasts of calli derived from some individuals divided several times in the modified 8p medium, but never formed colonies. This phenomenon was observed of all 4 cultivars. Therefore, the colony formation also appeared to depend upon the genotype of the plants in all cultivars. The formed colonies grew into calli on the modified B 5 medium. When calli of early subculture were used for the protoplast isolation, the adventitious embryos were formed from the subsequent callus culture on the modified Blaydes medium. The frequencies of the adventitious embryo formation appeared to be higher in Rangelander and Rambler than those in the other two cultivars. These embryos formed from protoplast culture also germinated and developed into complete plantlets on the B 5 medium without growth regulators (**Fig. 1-F**). Most of the protoplasts isolated from 5-7 subcultured calli, however, lost their totipotency. On the other hand, the callus lines which maintained the ability of embryogenesis after 5-7 subcultures also produced the adventitious embryos in protoplast culture in which the protoplasts were isolated from 5-7 subcultured calli.

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≪和文要約≫

# アルファルファ，*Medicago sativa* L.，のカルスおよびプロトプラストからの植物体再生における変異

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アルファルファ，*Medicago sativa* L.，の4品種の胚軸由来カルスからの不定胚および植物体再生能力について検討した．カルス誘導，不定胚形成のための前培養，不定胚形成および発芽のために Brown と Atanassov (1985) の方法に従ってそれぞれ異なる4種類の培地を用いた．植物体再生能力は品種によって，また，品種内の個体によって異なっていた．約1カ月ごとに 5〜7 回継代培養したカルスからの不定胚形成能力は急速に低下した．また，胚軸由来カルスから単離したプロトプラストからの植物体再生も試みた．新鮮培地に継代してから 2〜3 週間以内のカルスを4% セルラーゼ“オノズカ” RS，1% マセロザイム R-10，0.2% ペクトリアーゼ Y-23，0.7 M マンニトール液で1時間 28℃ で振盪処理してプロトプラストを単離した．このプロトプラストを Kao と Michayluk の改良 8p 培地（ゼアチンの代わりにベンジルアデニンを加え，ココナットミルクを除いたもの）で培養すると約2カ月でコロニーが形成された．しかし，このコロニー形成能力においても品種および個体間に差異が認められた．このコロニーからカルスを得，胚軸由来カルスと同様の培養方法で植物体を再生することができた．