식물학회지 제32권 제4호
Korean J. Bot. 32(4) : 313-322(1989)

# Induction of Multi Shoots and Plant Regeneration From Protoplasts of Alfalfa(*Medicago sativa* L.)

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# 알팔파(*Medicago sativa* L.)의 原形質體로부터 多莖 誘導와 植物體의 再分化

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## ABSTRACT

A system was established for induction of multi-shoots and plant regeneration from mesophyll protoplasts of alfalfa, *Medicago sativa* L. cv. Vernal. Different hormonal effects were tested at each step of protoplast culture, i.e. cell division in modified Kao's liquid medium (K566-7). calli formation on SH semi solid medium, and multi-shoot regeneration from calli on SHa and SHb solid media. Frequency of multi-shoots and plant regeneration was affected by various combinations of phytohormones in final step. The evaluation of multi-shoots induction systems via protoplast culture was discused.

## INTRODUCTION

Plant regeneration via somatic embryogenesis can be efficiently achieved only in relatively few plant species like carrot (Kameya and Uchimiya, 1972), caraway (ammirato *et al.*, 1977, 1983) and tobacco (Cocking, 1960;Bajaj, 1974;Gill *et al.*, 1979).
Reports on plant regeneration from protoplasts of leguminous plants are limited, although some progress has been made in the last decade(Lee et al., 1981, 1985; Xu *et al.*, 1982; Kim et al., 1983; Grant, 1984; Li *et al.*, 1985; Crepy *et al.*, 1986; Ghazi *et al.*, 1986; Saxena and Gill, 1987; Choi *et al.*, 1988). Among these, alfalfa and sonbean have been studied most extensibly.

Several groups reported that somatic embryos induced from the mesophyll protoplasts have developed into whole plant in alfalfa (Santos *et al.*, 1980; Kao and Michayluk, 1980; Johnson *et al.*, 1981; Atanassov and Daniel, 1984; Nagarajan *et al.*, 1986). In these reports on regeneration, we observed that the quantitative control by hormonal combinations of regeneration medium had profound effects on morphogenesis. However, these results are not giving full information on plant regeneration of alfalfa.

Therefore, here we have established a procedure of plant regeneration of alfalfa (*Medicago sativa* L. cv. Vernal) by obtaining multi shoots from protoplasts via somatic embryogenesis. The procedure induced alternative method of each culturing stages and required two steps culture for whole plant regeneration. Also we discussed the evaluation of multi shoots induction systems via protoplasts culture on the propagation of large number of leguminous plants using this procedure and culture systems.

## MATERIAL AND METHODS

Alfalfa plants, *Medicago sativa* L. cv. Vernal, were grown from seeds in the constant growth chamber at 25℃ and the 2nd or 3rd branches were used for protoplasts isolation. Enzyme mixture was composed of 2.0% Cellulase 'Onozuka' R-10 (Yakult Honsba Co., LTD, Tokyo, Japan) and 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co., LTD, Tokyo, Japan) in 0.6M mannitol solution, adjusted to pH 5.8 with 0.5N KOH.

The general scheme used for protoplasts culture and induction of multi-shoots was summarized in Fig. 1. Micro calli (2~3 mm diameter) derived from protoplasts on SH semi solid medium containing 0.05mg/1 NAA and 0.5mg/l BAP were transferred onto SHa solid medium containing 10mgl 2,4-D and 1.0mg/l Kinetin for production multiple embryogenic callus and cultured at 25 with a light intensity of 2000 lux (16h day length). When the multiple embryogenic calluses reached the size of 1.0~1.5cm in dimeter, they were transferred on to the SHb solid medium containing 0.1mg/l ABA plus either 0.1 mgl Zeatin or $GA_3$ for multi-shoot regeneration were also tested(Schenk and Hidebrandt, 1972; Saunders and Bingham, 1975; Ammirato, 1977, 1983; Walker *et al.,* 1979; Kao and Michayluk,, 1980; Santos *et*

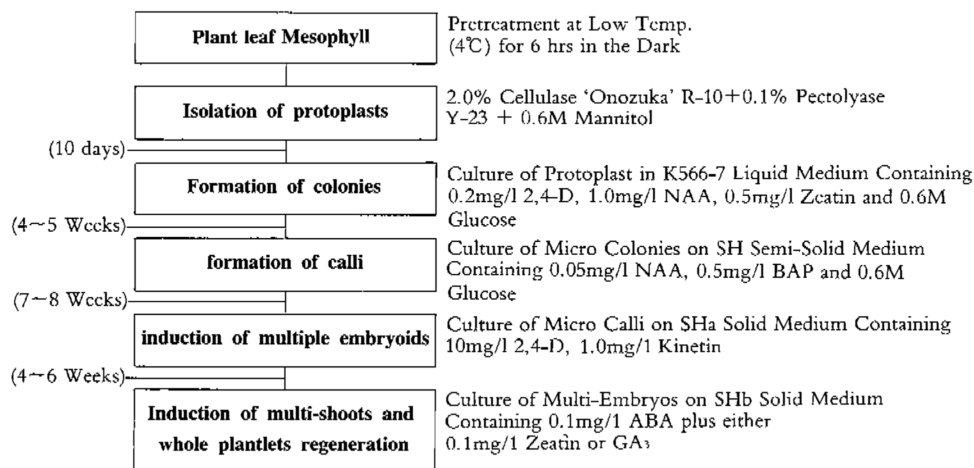


**Fig. 1.** Schematic diagram of procedure for induction of multi-shoots and plant regeneration from mesophyll protoplasts of *Medicago sativa* L. cv. Vernal.

*al.*, 1980; Johnson *et al.*, 1981; Arcioni *et al.*, 1982; Atanasov and Daniel, 1984; Ghazi *et al.*, 1986; Nagarajan *et al.*, 1986; Nishio *et al.*, 1987; Tsai, 1988).

After one month of culture, the number of calluses with regenerated multi-shoots was scored. Multi-shoots produced roots in this medium were transplanted into soil in small pots. pH of all media adjusted prior to autoclaving.

## RESULTS AND DISCUSSION

The obtained protoplasts were healthy, having viability sustained divisions (Fig. 2a). First cell division was found at 24~36h(Fig. 2b) and cell shape was changed from spherical to oval

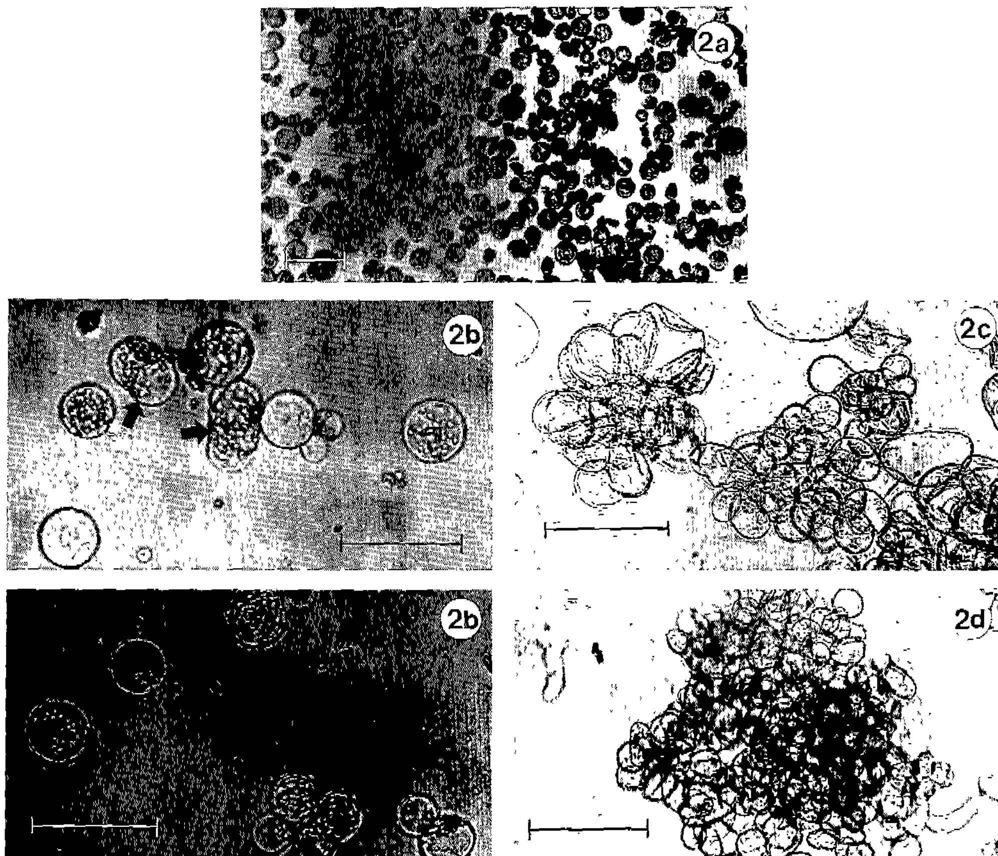


**Fig. 2.** Leaf derived protoplasts of *Medicago sativa L.* cv. Vernal with the enzyme solution of 2.0% Cellulase 'Onozuka' R-10, 0.1% Pectolyase Y-23 and 0.6M Mannitol (a), and various stages in division of the isolated protoplast in K566-7 liquid medium (b~d). a. Freshly isolated mesophyll protoplasts after 4.5 hrs in the enzyme solution. b. First cell division observed within 24~36 h of culture in K566-7 liquid medium. c. Cell clusters obtained within 48 h of culture. d. Cell colonies arising after 10 days of culture. Scale bars represent 100$\mu$m in a; 200$\mu$m in b, c; 400$\mu$m in d.

or slightly elongated form in modified Kao's liquid(disigned K566-7) medium. Afterward, cell division ensued in quick succesion, resulting in the formation of 6~8 celled(Fig. 2c), and colonies (Fig. 2d). The survival rate and division efficiency of surviving protoplasts at 7 days were averaged 50% and 34%(data not shown). These results are similar to the case described by other groups(Uchimiya and Murshige, 1974, 1976; Johnson *et al.*, 1981; Arcioni *et al.*, 1982; Lee *et al.*, 1985; Sticklen *et al.*, 1986).

Plating the protoplasts to directly onto agar media was tired several times (Nagata and Takebe, 1971), but the protoplasts were not growing in this method. Substitution of agar to agarose (Shillito *et at.*, 1983 : Holbrok et al., 1985) also had no effect (data not shown). Then micro colonies reaching the size 400~600$\mu$m in diameter, transferred onto the SH semi solid medium designated by Schenk and Hidebrandt(1972) after 10 days of culture in the liquid

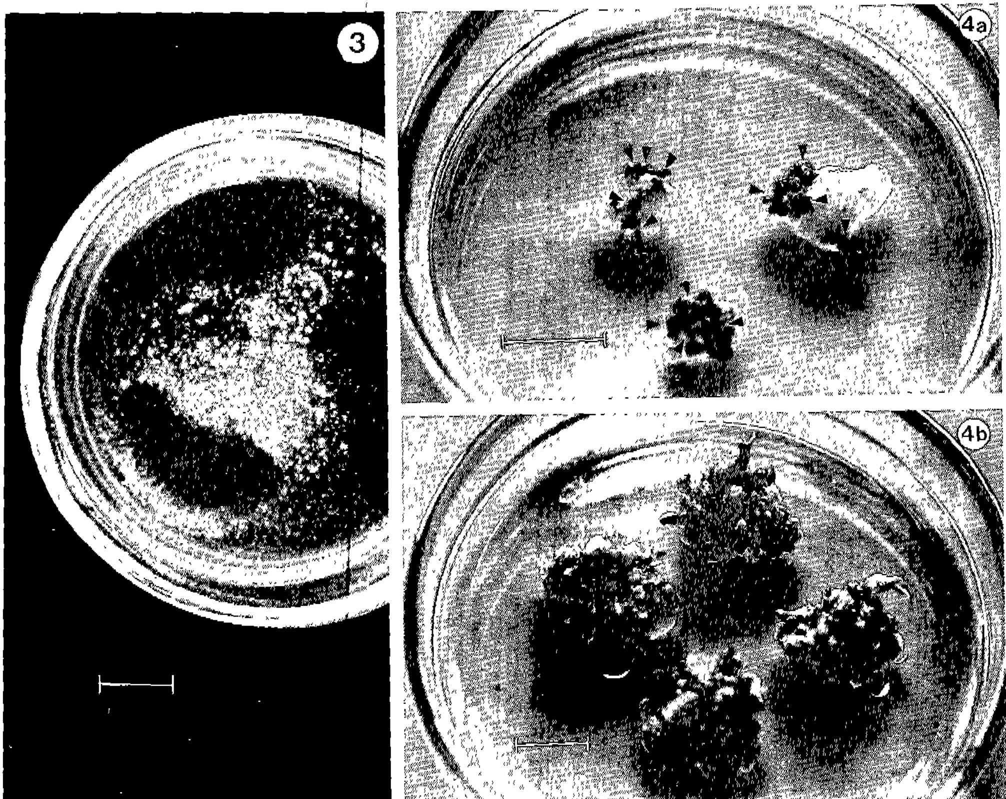


**Fig. 3.** Micro calli formation (2~3mm) after 4~5 weeks of culture *in* SH semi solid medium. Scale bar represents 1.0 cm.

**Fig. 4.** Proembryos formation(arrows) and multiple embryogenesis at the green region of calli surface on SHa solid medium. a. Proembryos formation at the calli surface after one month of inoculation on SHa solid medium. b. Multiple embryos developing a green region after 7~8 weeks of culture. Scale bars represent 1.0cm in a,b.

medium, were grown to about 2~3mm in diameter within 3~4 weeks (Fig.3). When these calli were transferred onto the SHa solid medium, multiple embryos were excellently formed from the calli placed on the media containing 10mg/l 2.4-D and 1.0mg/l Kinetin (Table 1), but shoot regeneration was negligible. Therefore, these multiple embryos developing a greenish region on the SHa solid media (Fig. 4a, b) were transferred onto SHb solid media.

The freuqency of the multi-shoot formation on the SHb solid medium during the period from 28 to 35 days after culturing was, in averaging, 10% without growth regulators as a control and were over 50% on 0.1mg/l ABA plus either 0.1mg/l Zeation or $GA_3$(Table 2). However, some of them were produced only roots (Zeatin+ABA+$GA_3$, Zeatin+ABA, ABA +$GA_3$, Zeatin and $GA_3$) or only shoots (Control, Zeatin $GA_3$ and ABA) when cultured on same media with different combinations of phytohormone (Fig. 5a). Among these combinations, whole plant regeneration was obtained in only four kinds of combination;Zeatin+ABA, ABA+$GA_3$, Zeatin and $GA_3$ (Fig. 5b). The maximum grade of whole plants induced was over 70% on SHb solid medium, plantlets are developed approximatly 12~13 cm long (6~7cm including roots). It was suitable size for transplanting to soil where they continued to develop in a similar fashion to normal seedlings in the growth chamber (Fig. 6).

Our results can be compared with previouss reports, the process of multi-shoot induction from protoplasts to composed with three steps, (1) protoplasts to micro colonies in K566-7

**Table 1.** Effect of 2.4-D and kinetin of SHa solid medium on multiple embryogenesis from micro calli. Efficiency of multiple embryogenesis can be expressed as ++++; excellent (〉80%), +++; very good (>70%), ++; good (>50~60%), +; fair (>30~50%), −; poor (<30%).

| Kinetin (mg/l) | 2.4-D(mg/l) | | | |
|---|---|---|---|---|
| | 1.0 | 5.0 | 10.0 | 15.0 |
| 0.1 | − | − | + | − |
| 0.5 | − | + | +++ | − |
| 1.0 | − | ++ | ++++ | + |
| 5.0 | − | − | ++ | − |

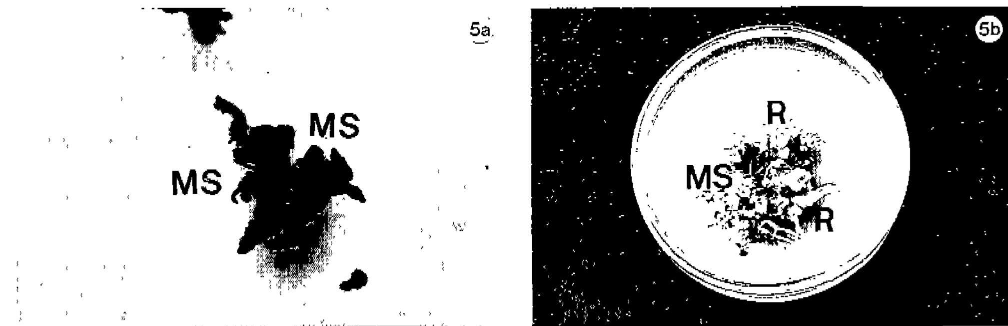


**Fig. 5.** Formation of multi shoots (a) and whole plant regeneration (b) from multiple embryos on SHb solid meium (R : Roots, MS : Multi-Shoots).

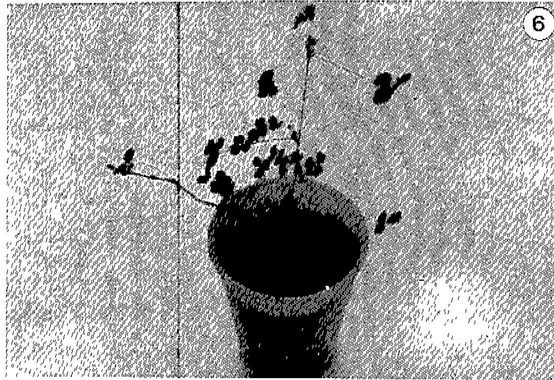


**Fig. 6.** Typical plants of transplanting to small pots with vermiculate containing nutrients.

**Table 2.** Effects of phytohormones on the efficiency of organogenesis. Mulit-shoots and roots were developed on the SHb solid medium containing 0.1 mg/l ABA plus either 0.1 mg/l Zeatin or $GA_3$ for 28~35 days from multiple embryogenesis which were formed on SHa solid medium.

| Combination of Phytohormones on SHb solid medium (0.1 mg/1) | Efficiency of organogenesis(%)* | | |
|---|---|---|---|
| | Shooting | Rooting | Plantlets |
| No growth regulator (Control) | 10 | — | — |
| Zeatin+ABA+$GA_3$ | 60 | 15 | — |
| Zeain+ABA | 70 | 20 | 20 |
| ZeatinGA$_3$ | 55 | — | — |
| ABA+$GA_3$ | 60 | 20 | 15 |
| Zeatin | 75 | 10 | 20 |
| ABA | 55 | — | — |
| $GA_3$ | 50 | 25 | 20 |

Percentages are the mean value of replication.

**Table 3.** Effects of cytokinins on the frequency of embryogenesis. Embryogenesis frequency was measured with 3 microcalli cultured in 6 cm diameter peridish for 7~8 weeks on SHa solid medium.

| Hormonal Combination | Frequency of embryogenesis(%)* | Mean No. of mature embryos per petri dish |
|---|---|---|
| 10mg/l 2,4-D<br>1mg/l kinetin | 82.6±4.54 | 26.8 |
| 10mg/l 2,4-D<br>1mg/l BA | 52.6±3.26 | 7.3 |
| 10mg/l 2,4-D<br>1mg/l 2ip | 50.7±6.23 | 14.6 |
| 10mg/l 2,4-D<br>1mg/l Zeatin | 67.6± 4.19 | 9.3 |

*Percentages are the mean ± S.D. of 3 determination.

liquid media for 10 days, (2) micro colonies to micro calli on SH semi solid agar media for one month, (3) multi-shoots and plant regeneration from calli on SHa and SHb solid agar media for three months.

During the observation of an optimum hormonal combination in each step, it was found that the frequency of multi-shoot induction was highly influenced by the hormonal constituent in the SHa and SHb solid media (the final step). It was the key factor obtaining multi-shoots in large quantity. Finding that this procedures provided conditions gradually adaptative for plant regeneration from embryogenic calluses using the SH medium composition, is agreed with the earlier results of privious groups (Saunders and Bingham, 1975; Walker *et al.*, 1979; White, 1984; Stuart and Strickland, 1984; Strickland *et al.*, 1987; Nishio *et al.*, 1987; Tsai, 1988).

Combinations of high concentration of 2,4-D (10mg/1) and low concentration of cytokinins (1.0mg/1) on SHa medium gave the highest multiple embryogenesis, and those of low concentration of phytohormones (0.1 mg/l ABA plus either 0.1 mg/l Zeatin or $GA_3$) on SHb medium resulted in the development of multiple embryogenic calluses with high capacity of multi-shoot induction. However, combination of low concentration of 2,4-D on SHa medium and those of high concentration of phytohormones on SHb solid medium resulted in the death of calli or the development of embryogenic calluses with low regeneration capacity. (Table 2 and 3).

As Saunders and Bingham (1975), Walker *et al.* (1979), Kao and Michayluk (1981) established that the shoot formation in alfalfa is promoted by exposure to relatively high 2,4-D and low Kinetin concentrations. Also, Santos *et al.* (1980), Arcioni *et al.* (1982) and Ghazi *et al.* (1986) reported the similar concentration of 2,4-D and Kinetin for somatic embryogenesis in leguminous plants callus. Addicott and Lyon (1969), Shabde and Murashige (1977), Ammirato (1977, 1983), Shepard (1980, 1982) and Ghazi *et al.* (1986) suggested that induction of organogenesis in tisue cultures requires the low concentration of ABA plus either low concentration of Zeatin or $GA_3$.

From these results, it is clear that high concentration of auxins and low concentration of cytokinins on SHa solid media were esential for the embryogenesis, and low concentration of phytohormones on SHb solid medium was necesary for the efficient shoot inducttion. Although these results obtained by them, our research improved the step induction method used to induce organogenesis from the protoplasts.

High fraction (50%) of calli was differentiated by improving this procedures and nearly 70% of the multiple embryos produced multi-shoots. This efficiency is higher than that reported by previous groups. This procedures represents an important step toward the development of a program of somatic hybridization and protoplast technology. It is possible to propagate large number of leguminous plants using these conditions and culture systems.

## 摘 要

알팔파(*Medicago sativa* L. cv. Vernal)의 엽육조직으로부터 원형질체를 분리하여 다경유도와 식물체의 재분화에 필요한 실험조건을 조사하였다. 그 결과 단계별 선택배지 상에서 서로 다른 호르몬의 조합과 배지형태에 따라 원형질체로부터 다경유도와 다수의 식물체를 얻을 수 있었다. 즉, 첫 번째 단계에서 Kao의 액체 개변배지 (K566-7)로써 세포배양을 시도하고, 두번째 단계로 SH 고체배지 상에서 칼루스를 형성시켰으며, 세번째 단계로 SHa와 SHb 고체배지 상에서 다경의 유도와 식물체의 재분화를 효과적으로 수행할 수 있었다.

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(Received sept. 16, 1989)