# The Protein and Isozyme Patterns During *in vitro* Plant Regeneration of Yooja (*Citrus junos Sieb.*) and Trifoliate Orange (*Poncirus trifoliata Rafin.*)

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## ABSTRACT

**In this study, plant regeneration through *in vitro* culture from plantlet stems of Yooja (*C. junos* Sieb.) and trifoliate orange (*P. trifoliata Rafin.*) was attempted to make mass-production system of virus-free plants having the same genotype with mother plant. In order to investigate physiological change depending on the developmental stage of plant regeneration, the changes of total protein, peroxidase and esterase activity and their isozyme patterns as well were examined in 1/2 MS medium.**

**The results are as follows :**

**1. The MS medium for the optimal callus induction and shoot formation was utilized. The medium was supplemented either with 2,4-D and Kinetin or with BA and NAA. The optimal concentrations were the combination of 1.0㎎/ 2,4-D + 0.3㎎/ Kinetin and 1.0㎎/ BA + 0.3 ㎎/ NAA in callus induction and shoot formation, respectively.**

**2. For the plant regeneration from somatic embryos, 1/2 MS medium was used with supplements of growth regulators (free, 1.0㎎/ IBA + 1.0㎎/ BA , 0.5㎎/ IBA + 0.5㎎/ BA). Shooting and rooting were the best in the treatment of 0.5㎎/ IBA and 0.5㎎/ BA combination.**

**3. The total protein content has a tendency of increase with the developmental stage of embryo, but it was decreased at the plantlet. Also it was the highest at 8 and 6 weeks stage in *C. junos* Sieb. and *P. trifoliata* Rafin, respectively. In the SDS-PAGE pattern of protein, *C. junos* Sieb. showed bands of 29.0 and 40kDa at 10 weeks. The 45, 66 and 97.4 kDa bands at 10 weeks of culture were shown in *P. trifoliata* Rafin.**

**4. The highest esterase activity was shown at the 6 and 8 weeks of culture in *C. junos* Sieb. and *P. trifoliata* Rafin., respectively.**

**5. Esterase isozyme patterns were shown difference according to the developmental stage. In *C. junos* Sieb. a new band was observed at pI 7.7 following 4 weeks culture. On the other hand, new bands in *P. trifoliata* Rafin. were observed at pI 7.5～6.5 following 4 and 6 weeks culture, respectively.**

***Key Words :****Citrus junos* Sieb., *Poncirus trifoliata* Rafin., Protein and isozyme pattern, Plant regeneration

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## INTRODUCTION

Many plants undergo changes in plant regulator during growth and development and isozyme expression (Coppens and Decimate 1990). The changes of activity and isozyme pattern are correlated with growth, development and plant growth regulation. The analysis of isozymes at different stages of culture was used to variable tools in physiological, genetic and biochemical studies underlying the process of plant regeneration and differentiation (Coppens and Gillis, 1987).

Protein or isozyme patterns are widely used to distinguish closely related plant varieties, species or cultivars. The esterase isozyme refers to a group of enzymes, inclusive a host of ester hydrolase (Kidambi, 1990). Combination and refinement of analyzed isozyme by isoelectric focusing provide a reliable marker system for organigenesis and embryogenesis of plants in the callus. The developmentaly regulated isozyme system of esterase to distinguish between embryogenesis and shoot-forming in maize tissue cultures was used by Everette and his coworkers(1985). Isozyme system of esterase is known as a biochemical maker between shoot forming maize tissue and embryogenic culture (Everette *et al*, 1985 : Coppens and Gillis, 1987).

Plant regeneration from shoot tip, immature ovules, cotyledon, hypocotyl and leaf of Trivita orange(*C. sinensis*), Naval orange(*C. sinensis*), Sweet orange(*C. sinensis*), Trifoliate orange(*Poncirus trifoliata*), *Citrus junos* Osbeck., *Citrus junos* Sieb. and *Citrus junos* Blanco. in citrus has been reported (Kobayashi *et al*, 1987 ; Kobayashi *et al*, 1990, Shinozaki *et al*, 1992 : Hiramatsu *et al*, 1987 ; Ikeda and Yoshida, 1993 ; Song *et al*, 1991). Also, our laboratory has achieved plant regeneration from hypocortyl of immature seed of *C. junos* Sieb. (Park *et al*, 1995). In addition, isozyme analysis is very useful for the studies of classification and genetics. Therefore we has carried out a series of techniques used by Hirai and his coworkers in this experiment (Hirai *et al*., 1986; Hirai and Kajiura, 1987, Kensuke, 1993).

The objective of this study, to clarify the different esterase isozyme and protein banding patterns in the variable stages of the plant tissue culture, and the most efficient system from somatic embryogenesis and plant regeneration by embryogenic callus from the hypocotyl region of immature embryo in *C. junos* Sieb. and finally to get the fundamental information of genetic analysis about a system phylogeny.

## MATERIALS AND METHODS

### Plant Materials

Mature seeds of Yooja (*C. junos* Sieb.) and trifoliate orange (*P. trifoliata* Rafin.) were obtained in the farm of Kokum island in November (Fig. 1A. 1B). Seeds were sterilzed with 70%(V/V) ethanol for 10min and 5%(W/V) NaOCl for 15min and rinsed 3 times in sterilled water. The stem used in the tissue culture was a part of developmental plantlet germinated *in vitro* from sterilized seed.

### Callus Induction and Plant Regeneration

For callus induction, stem pieces were cultured on MS medium supplemented with different concentration of various plant growth regulators [0.1 ~ 1.5 mg/L 2,4-D + 0.1 ~ 0.4 mg/L kinetin KIN), 0.1 ~ 1.5 mg/L KIN + 0.1 ~ 0.4 mg/L 2,4-D, 0.1 ~ 1.5 mg/L BA + 0.1 ~ 0.4 mg/L NAA, 0.1 ~ 0.4 mg/L BA + 0.1 ~ 1.5 mg/L NAA] in vitro. The medium was adjusted to pH 5.8 before autoclave. And these were incubated in a thermostatically controlled room to maintain a temperature of 25 2 under white flourescent light for 16h in 2,000 Lux and dark period for 8h.

After callus induction, the embryogenic calli were cultured onto MS medium without any growth

Table 1. Effect of 2, 4-D and KIN for shooting and callus induction from stem and leaf segment for 8 weeks in *Citrus junos* SIEB.

| 2,4-D/KIN concentration(㎎/ ) | leaf | | stem | |
|---|---|---|---|---|
| | callus induction | shooting | induction | callus shooting |
| 0.1/0.1 | * | # | * | # |
| 0.5/0.2 | ** | # | ** | # |
| 0.2/0.5 | ** | ** | ** | ** |
| 1.0/0.3 | **** | # | **** | # |
| 0.3/1.0 | *** | *** | *** | *** |
| 1.5/0.4 | *** | # | *** | # |
| 0.4/1.5 | *** | *** | *** | *** |

Degree of callus and shoot development ; # : none * : rare, ** : moderate, *** : good, **** : excellent.

Table 2. Effect of plant growth regulators for shooting and callus induction from stem and leaf segment for 8 weeks in *Citrus junos* SIEB.

| 2,4-D/KIN concentration(㎎/ ) | leaf | | stem | |
|---|---|---|---|---|
| | callus induction | shooting | induction | callus shooting |
| 0.1/0.2 | * | * | * | * |
| 0.2/0.1 | * | * | * | * |
| 0.5/0.2 | ** | ** | ** | ** |
| 0.2/0.5 | ** | ** | ** | ** |
| 1.0/0.3 | *** | **** | *** | **** |
| 0.3/1.0 | *** | *** | *** | *** |
| 1.5/0.4 | **** | **** | **** | **** |
| 0.4/1.5 | *** | *** | *** | *** |

Degree of callus and shoot development ; # : none * : rare, ** : moderate, *** : good, **** : excellent.

Table 3. Effect of BA and NAA for shooting and callus induction from stem and leaf segment for 8 weeks in *Poncirus trifoliata* RAFIN.

| 2,4-D/KIN concentration(㎎/ ) | leaf | | stem | |
|---|---|---|---|---|
| | callus induction | shooting | induction | callus shooting |
| 0.1/0.1 | * | * | * | * |
| 0.5/0.2 | ** | ** | ** | ** |
| 0.2/0.5 | ** | ** | ** | ** |
| 1.0/0.3 | **** | **** | **** | **** |
| 0.3/1.0 | *** | *** | *** | *** |
| 1.5/0.4 | *** | *** | *** | *** |
| 0.4/1.5 | *** | *** | *** | *** |

Degree of callus and shoot development ; # : none * : rare, ** : moderate, *** : good, **** : excellent.

regulators and about 7㎝ young plant was observed after 23 weeks culture.

**Protein Analysis**

***Protein extraction*** : About 1g of fresh plantlet was homogeized with 5㎖ of 50mM tris-HCl buffer (pH 7.2) at 4 . The homogenate was centrifuged for 30min at 13,000g and supernatant was used for protein alalysis.

Measurement of protein content : Protein was measured by the method of Bio-Rad protein assay.

Table 4. Effect of 2,4-D and KIN shooting and callus induction from stem and leaf segment for 8 weeks in *Poncirus trifoliata* RAFIN.

| 2,4-D/KIN concentration(㎎/ ) | leaf | | stem | |
|---|---|---|---|---|
| | callus induction | shooting | induction | callus shooting |
| 0.1/0.1 | * | * | * | * |
| 0.5/0.2 | ** | ** | ** | ** |
| 0.2/0.5 | ** | ** | ** | ** |
| 1.0/0.3 | **** | **** | **** | **** |
| 0.3/1.0 | *** | *** | *** | *** |
| 1.5/0.4 | *** | *** | *** | *** |
| 0.4/1.5 | *** | *** | *** | *** |

Degree of callus and shoot development ; # : none * : rare, ** : moderate, *** : good, **** : excellent.

Mixture of Bio-Rad dye(1㎖), DW(4㎖), and crude enzyme(5㎕) was treated at RT for 10min. Treated crude enzyme was measured by the absorbance at 595nm.

***SDS-PAGE*** : The molecular weight of protein was determined on the 12%(W/V) polyacrylamide gel by the method of Laemmli(1970). Electrophoresis was performed for 4h at 4 , 150V. Standard markers of Bio-Rad were the mixture of phosphorylase b (97.4kD), bovine serum albumin (66kD), egg albumin (45kD), carbonic anhydrase (31kD), trypsin inhibitor (21.5kD) and lysozyme (14.5kD).

**Analysis of Esterase and Peroxidase**

***Isozyme preparation*** : Esterase and peroxidase were prepared as described by Coppens and Gillis(1987) and Grison and Pilot(1985), respectively. In the esterase, about 1g tissue of fresh plantlet was homogenated with 100mM tris-HCl buffer (pH7.2) at 4 . The homogenate was centrifuged at 15,000g and supernatant was used for isozyme analysis.

In the case of peroxidase, 1g tissue of fresh plantlet was extracted by homogenization with 0.06mM phosphate buffer (pH 6.0) in medicine pestle. The homogenate was centrifuged at 18,000g, 4 for 15min and collected supernant was used crude enzyme.

***Activity measurement*** : Crude extract of esterase was added to 100mM phosphate buffer (pH6.0) with - $\alpha$ naphtyl acetate and Fast Blue RR Salt. Activity was tested by measurement of the absorbance at 290nm after 15min incubation at 30 . Crude peroxidase was reacted in 40mM phosphate buffer (pH 6.5) with 10mM guaicol and 10mM $H_2O_2$ at 30 for 5min. Activity was decided at OD 470nm.

***IEF*** : IEF were prepared as descried by Stegman and Park(1979). IEF was performed on 5% polyacrylamde gel containing 0.1% carrier ampholytes (Sigma) in the range of pH 3 ~ 10, 4 because of esterase.

Electrode strips were saturated with 0.01M $H_3PO_4$ as an anolyte and 0.2M NaOH as a catolyte. IEF standard proteins were used cytochrom c (pI 9.6), lentil lectine (pI 7.8, 8.0, 8.2), human hemoglobin c (pI 7.5), human hemoglobin a (pI 7.1), equine myoglobin(pI 7.0), human carbonic anhydrase(pI 6.5), bovine carbonic anhydrase(pI 6.0), $\beta$-lactoglobulin b(pI 5.1) and phycocyanin(pI 4.75).

***Activity staining*** : After electrophoresis, each gel was stained for enzyme activity. The staining procedure was stained by the method of Kidambi *et al*(1990) and Wetter and Dyck(1983). 20mg of $\alpha$-naphtyl acetate was dissolved in 0.5ml of acetone and the volume was made up to 100mM Tris-HCl buffer (pH 7.0). And then 40mg of Fast Blue RR Salt was dissolved by vigorous stirring. Gel was incubated at 37 for 15min. After staining ,the gel was rinsed and stored in 7% acetic acid.


**Fig. 1.** Yooja and trifoliata orange of mature fruits
A. Yooja(*C. junos* Sieb.) B. trifoliata orange (*P. trifoliata* Rafin.)

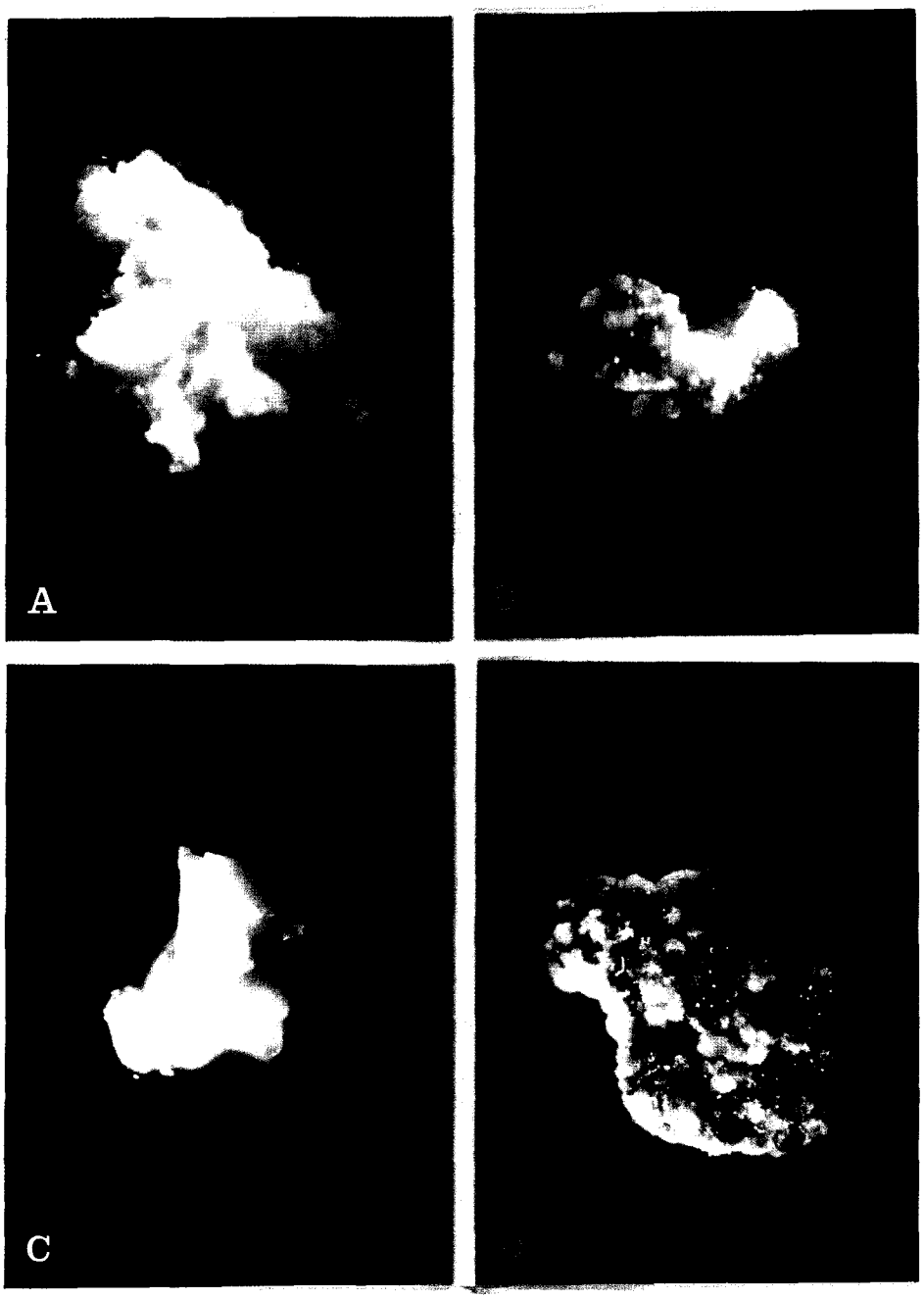


**Fig. 2.** Callus induction from stem for 8 weeks culture on MS orange and trifoliata orange.
A. Callus on MS medium with 2,4-D(1.0 mg/L) in yooja
B. Callus on MS medium with BA(1.0 mg/L) and NAA(0.3mg/L) in yooja
C. Callus on MS medium with 2,4-D(1.0 mg/L) and KIN(0.3mg/L) in trifoliata orange
D. Callus on MS medium with BA(1.0 mg/L) and NAA(0.3mg/L) in trifoliata orange

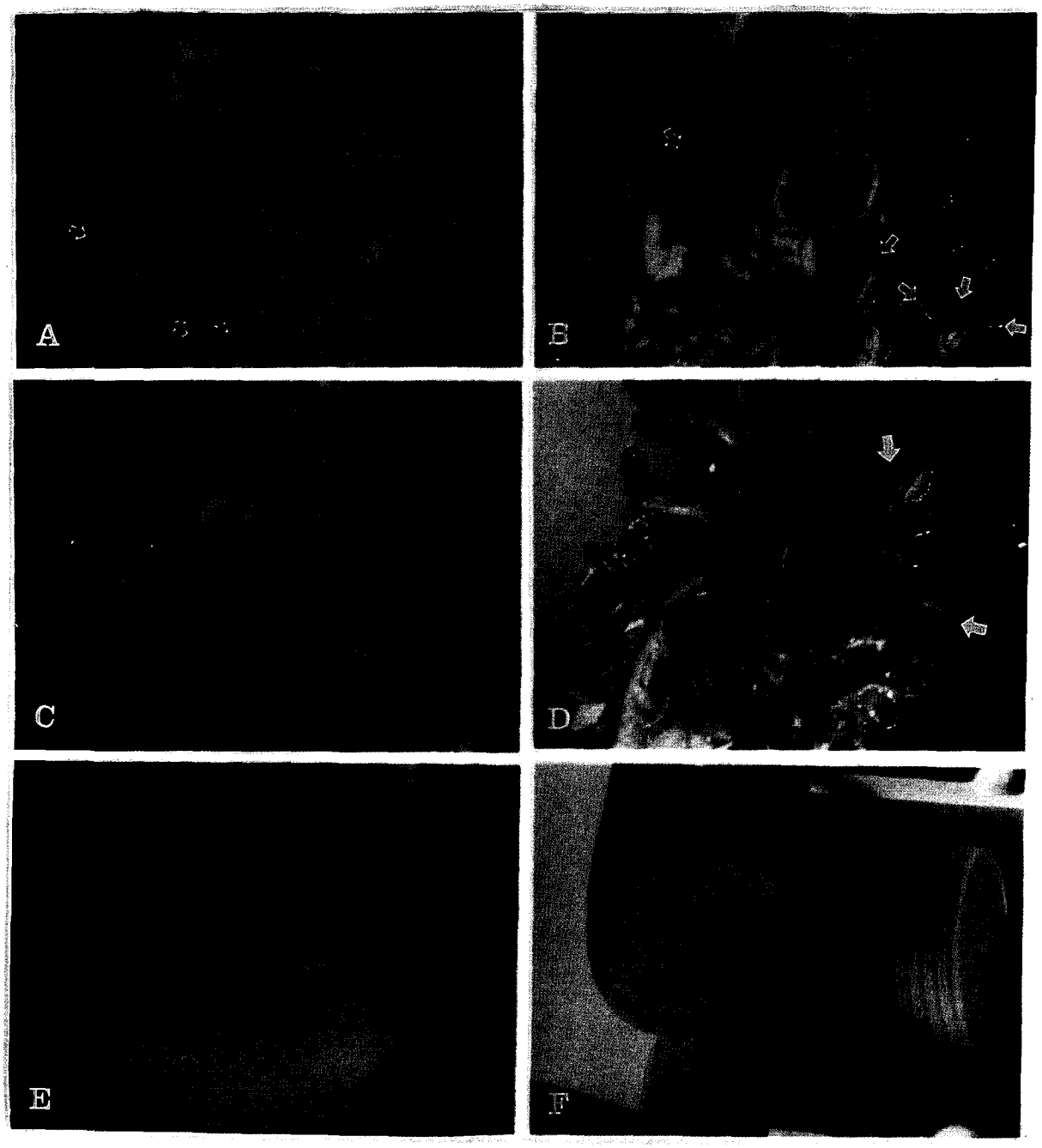


**Fig. 3.** (A-F) Plant regeneration on 1/2 MS medium in yooja
A. Shooting from globular-shaped embryo for 2 weeks culture
B. The shoot tip induced for 4 weeks culture
C. Leaf differenctiation from shoot tip for 6 weeks culture
D. The formation and differenctiationof stem for 8 weeks culture
E. Normal plantlet in 1/2 MS medium with IBA 0.5 mg/l +BA 0.5mg/l for 10 weeks culture
F. The growing plantlet for 15 weeks culture

Table 5. Effect of various concentration of IBA, BA on plantlet regeneration from the globular shaped somatic embryo in MS medium *in Citrus junos* SIEB.

| Growth regulator | Concentration(㎎/ ) | Plant regeneration |
|---|---|---|
| Free | - | # |
| IBA+BA | 0.5 + 0.5 | ## |
| IBA | 1.0 | ## |
| BA | 1.0 | ## |

*. Plant regeneration degree ; # : modrate ## : good ### : excellent

Table 6. Effect of various concentration of IBA, BA on plantlet regeneration from the globular shaped somatic embryo in 1/2 MS medium in *Citrus junos* SIEB.

| Growth regulator | Concentration(㎎/ ) | Plant regeneration |
|---|---|---|
| Free | - | # |
| IBA+BA | 0.5 + 0.5 | ### |
| IBA | 1.0 | # |
| BA | 1.0 | ## |

*. Plant regeneration degree ; # : modrate ## : good ### : excellent

## RESULTS AND DISCUSSION

**The Effects of Growth Regulator to Callus Induction**

To determine the most optimal concentration of growth regulator, MS medium was supplemented with various concentration of 2,4-D, NAA, BA, and KIN for callus induction and shooting.

Callus induction and shooting were the most effective (+++) in 2,4-D (1.0mg/L) / KIN (0.3mg/L) and BA (1.0mg/L) / NAA (0.3mg/L). Etiolated green and strong green shoot were induced in yooja, trifoliate orange, respectively (Table. 1). But callus and shoot were rarely formed in the 2,4-D (0.1mg/L) / KIN (0.1mg/L) and BA (0.1mg/L) / NAA (0.1mg/L) (Table 2). In the case of 2,4-D (1.5mg/L) / KIN (0.4mg/L), only callus was induced very good (+++) without shooting in yooja and while callus and shoot were induced very good (+++) in trifoliate orange. Also, the callus and shoot of yooja and trifoliate orange were moderate(++) in KIN (0.5mg/L) /2,4-D (0.2mg/L) and BA (0.2mg/L) /NAA (0.5mg/L), respectively. Green coloured shoots in yooja and trifoliate orange were induced very good(+++) in KIN (1.0mg/L) /2,4-D (0.3mg/L) (Table 3). In yooja and trifoliate orange, callus induction and shoot were better(+++) in KIN (1.5mg/L) and 2,4-D (0.4 mg/L), otherwise 0.3mg/L+1.0mg/L and 0.4mg/L+1.5mg/L were more good (+++) than 0.5mg/L +0.2mg/L and 0.2mg/L +0.5mg/L in BA/NAA.

In the case of BA (1.5mg/L) /NAA (0.4mg/L), callus induction and shoot of yooja were more excellent (++++) than those of trifoliate orange (Table 4).

As a results, cultured callus in 2,4-D/KIN (1.0mg/L

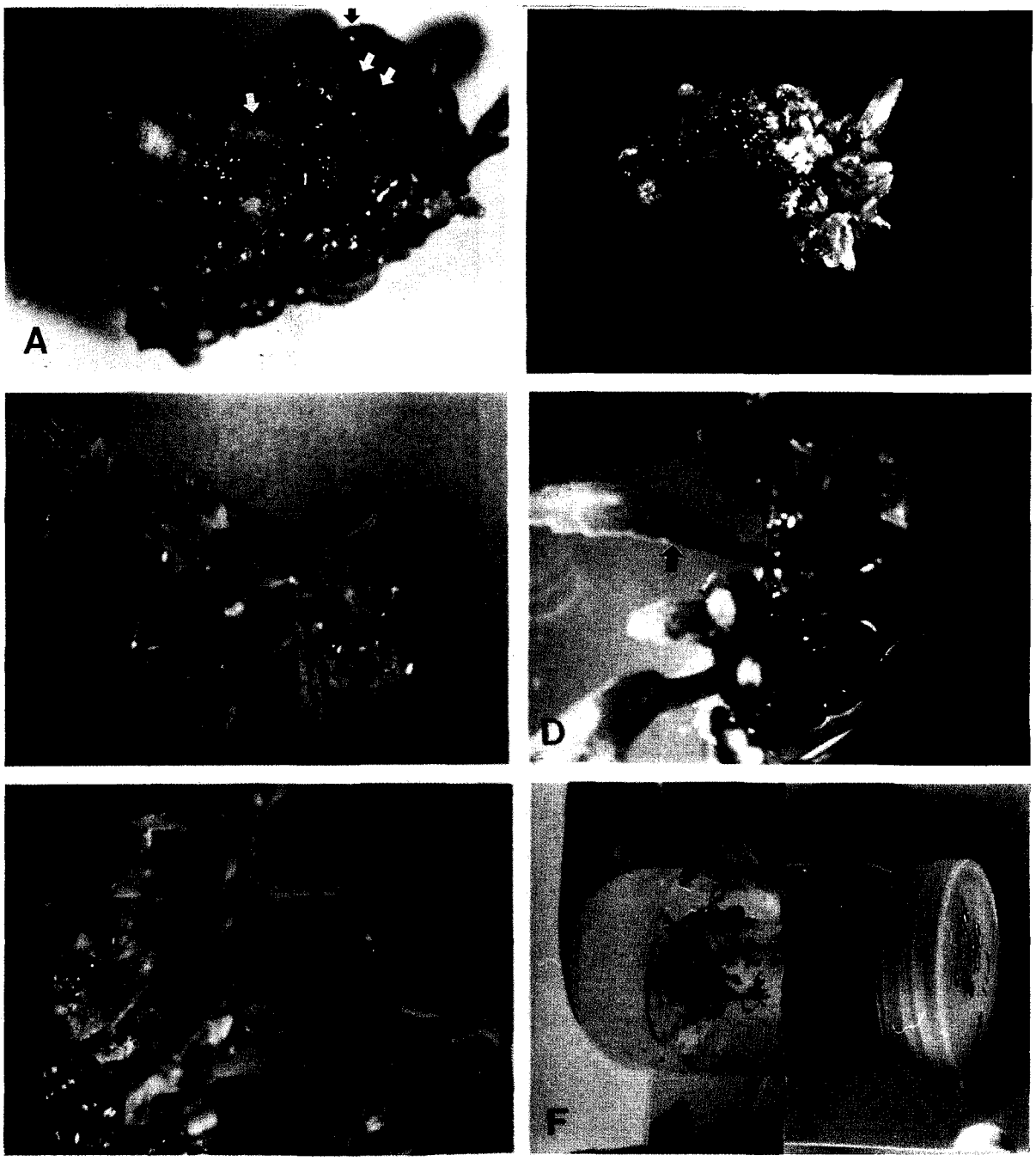


**Fig. 4.** (A-F) Plant regeneration on 1/2 MS medium in trifoliate orange.
A. Shooting from globular - shaped embryo for 2 weeks culture
B. The shooting tip induced for 4 weeks culture
C. The differentiation of the leaf from shoot tip for 6 weeks culture
D.The differentiation of the leaf and stem from shoot tip for 8 weeks culture
E. Normal plantlet for 10 weeks culture
F. The growing plantlet for 15 weeks culture

Table 7. Effect of various concentration of IBA, BA on plantlet regeneration from the globular shaped somatic embryo in MS medium in *Poncirus trifoliate* RAFIN.

| Growth regulator | Concentration(㎎/ ) | Plant regeneration |
|---|---|---|
| Free | - | # |
| IBA+BA | 0.5 + 0.5 | ## |
| IBA | 1.0 | ## |
| BA | 1.0 | ## |

*. Plant regeneration degree ; # : modrate ## : good ### : excellent

Table 8. Effect of various concentration of IBA, BA on plantlet regeneration from the globular shaped somatic embryo in 1/2MS medium in *Poncirus trifoliate* RAFIN.

| Growth regulator | Concentration(㎎/ ) | Plant regeneration |
|---|---|---|
| Free | - | # |
| IBA+BA | 0.5 + 0.5 | ### |
| IBA | 1.0 | # |
| BA | 1.0 | ## |

*. Plant regeneration degree ; # : modrate ## : good ### : excellent

+ 0.3mg/L) and BA/NAA (0.3mg/L + 1.0mg/L) was effectively induced more than that of KIN/2,4-D (1.0mg/L + 0.3mg/L) and BA/NAA (1.0mg/L + 0.3mg/L), respectively. However, in the high concentration of growth regulator, callus induction and shoot were more in trifoliate orange rather than Yooja. That is, callus was effectively induced in more 2,4-D than NAA and shooting was effectively in more BA than KIN.

**Plant Regeneration**

To regenerate plantlets callus for 8 weeks was cultured on MS and MS medium with various concentration of growth regulators[IBA(1.0, 0.5mg/L), BA(0.5, 1.0mg/L)] and free-growth regulators (Table5-8).

Plant regeneration was more in IBA (0.5mg/L) / BA (0.5mg/L) and free growth regulators than in IBA (1.0mg/L) / BA (1.0mg/L) (Table 5,7). Also rooting was more on MS than 1/2 MS medium in both of Yooja and trifoliate orange. Fig. 1 was yooja and trifoliate orange of mature fruits and Fig. 2 was callus induction from stem for 8 weeks culture on MS in orange and trifoliate orange. Shooting from the globular protrusion in yooja was started at 2 weeks culture, and it was more in IBA (0.5mg/L) / BA (0.5mg/L) than in the other hormone treatment(Fig. 3A). Tip was formed at 4 weeks culture and the differentiation of leaf and stem were started at 6 weeks culture (Fig. 3B,C). The development of main and side stem was excellent at 8 weeks culture with rooting(Fig. 3D). In particular rooting was induced rapidly on 1/2 MS medium supplemented with IBA / BA (0.5mg/L + 0.5mg/L). In the 1.0mg/L of IBA, rooting was good but the

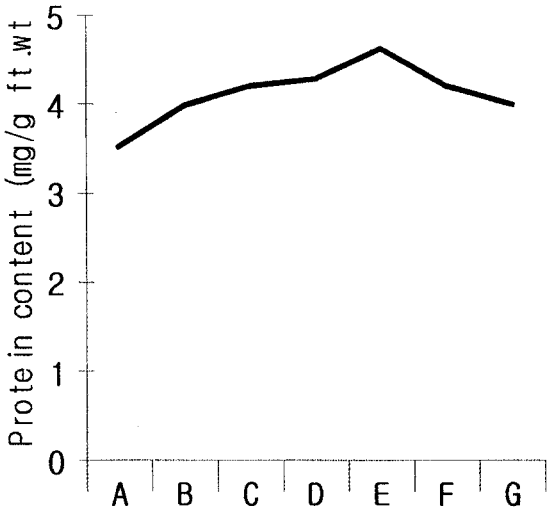


**Fig. 5.** Protein content of development stage in *Citrus junos* SIEB.
A, Callus; B, 2 weeks culture; C, 4 weeks culture;
D, 6 weeks culture; E, 8 weeks culture; F, 10 weeks culture; G, 15 weeks culture;

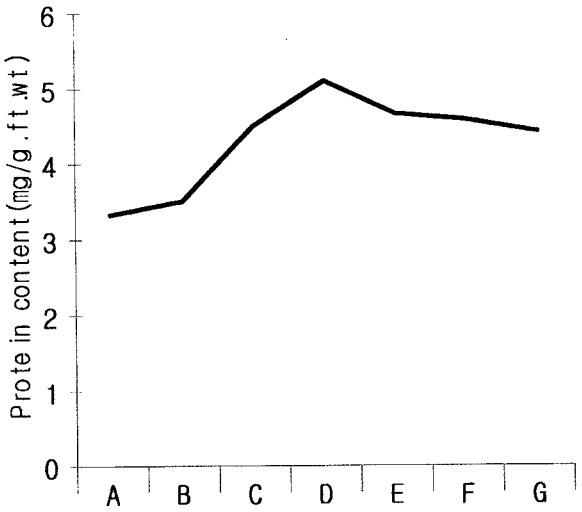


**Fig. 6.** Protein content of development stage in *Poncirus trifoliata* RAFIN.
A, Callus; B, 2 weeks culture; C, 4 weeks culture;
D, 6 weeks culture; E, 8 weeks culture; F, 10 weeks culture; G, 15 weeks culture;

development of leaf and stem was not good. At 10 weeks culture, plantlet was appeared and grown at 15 weeks culture (Fig. 3E,F).

In trifoliate orange, shooting appeared to be more dark green colour than yooja at 2 weeks culture (Fig. 4A). In trifoliate orange and yooja, differentiation of tip, leaf and stem were started at 4, 6 weeks culture, respectively (Fig.4B,C). Leaf and stem were differentiated prosperously after 8 weeks culture and appeared at 9 weeks culture (Fig.4D,E). Rooting was differentiated fairly on 1/2 MS medium with IBA (0.5mg/L) / BA (0.5mg/L) and IBA (1.0mg/L), respectively (Fig.4F). These results were similar to yooja. In the case of 1.0mg/L IBA, root was formed well but not good in leaf and stem. Also, plantlet was developed at 10 between 15 weeks culture similar to yooja.

**The Change of Protein Contents During Developmental Stage**

This research analyzed the change of protein contents according to developmental stage during plant regeneration.

The protein contents of yooja were measured from the prepared samples; callus, 2 (early shooting stage), 4 (formation of shoot tip), 6 (leaf differentiaton), 8 (mature stage of leaf differentiation), 10 (rooting stage), and 15 weeks culture (plantlet) (Fig5). The protein contents were gradually increased from 2 to 8 weeks culture and slowly decreased 10～15 weeks culture.

In addition the results of trifoliate orange were all most similar to yooja. protein contents of trifoliate orange were markedly increased 2～6 weeks culture and gradually decreased after 8 weeks culture (Fig. 6).

**The Change of Protein Patterns of SDS-PAGE in Developmental Stage**

This research observed band patterns of protein during plant regeneration from callus to 15 weeks

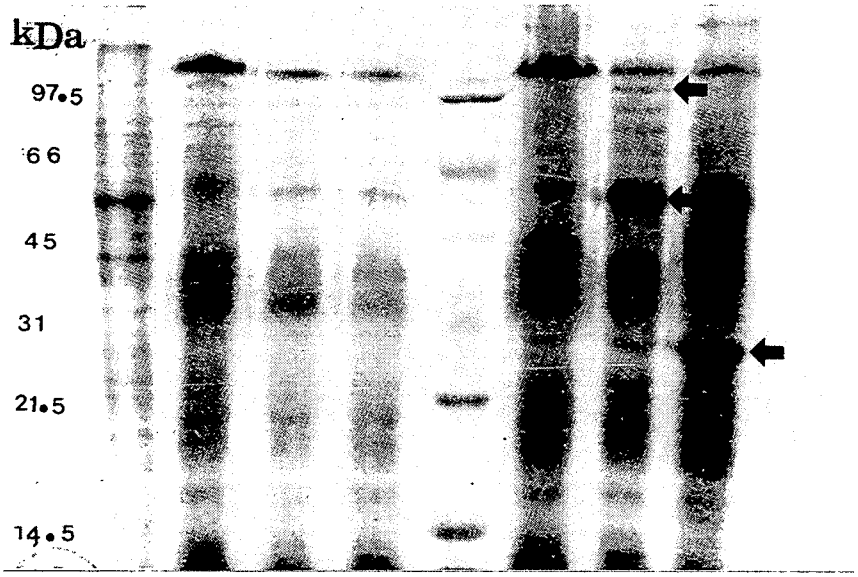


**Fig. 7** The protein patterns of the various stage of somatic embryo in yooja(*C.junos* SIEB)
A, Callus; B, 2weeks culture; C, 4 weeks culture; D, 6 weeks culture; E, 8 weeks culture;
F, 10 weeks culture; G, 15 weeks culture;

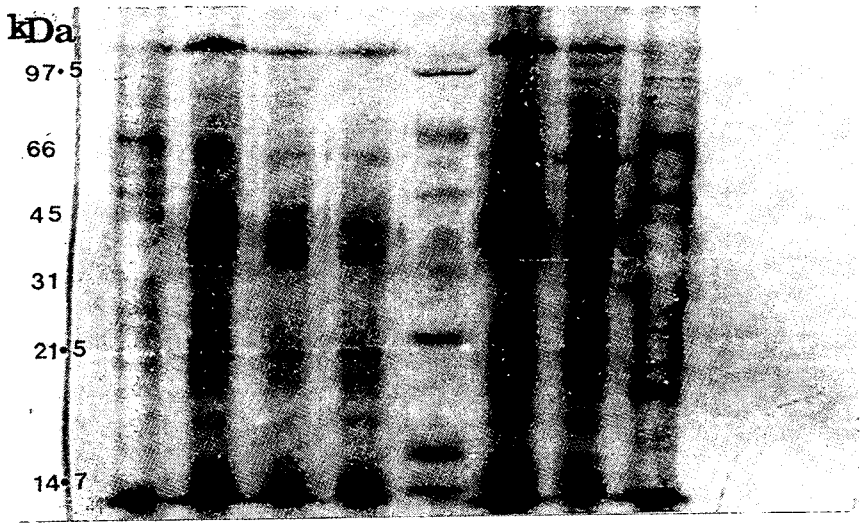


**Fig. 8.** The protein patterns of the various stage of somatic embryo in trifoliate orange (*P. trifoliata* RAFIN)
A, Callus; B, 2weeks culture; C, 4 weeks culture; D, 6 weeks culture; E, 8 weeks culture;
F, 10 weeks culture; G, 15 weeks culture;

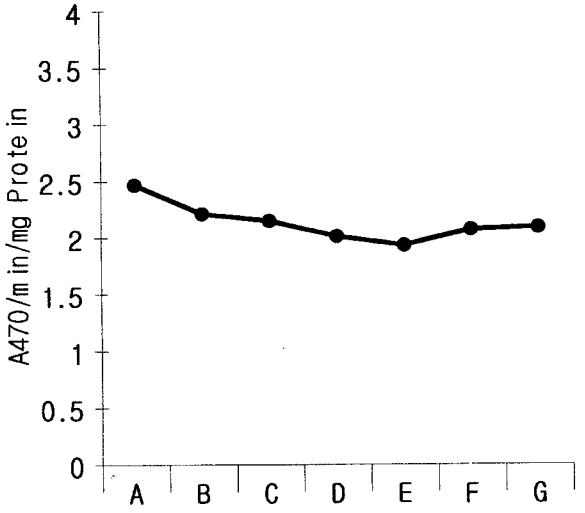


**Fig. 9.** Peroxidase activity in the developmental stage of somatic embryo in yooja.
A, Callus; B, 2 weeks culture; C, 4 weeks culture; D, 6 weeks culture; E, 8 weeks culture;
F, 10 weeks culture; G, 15 weeks culture.

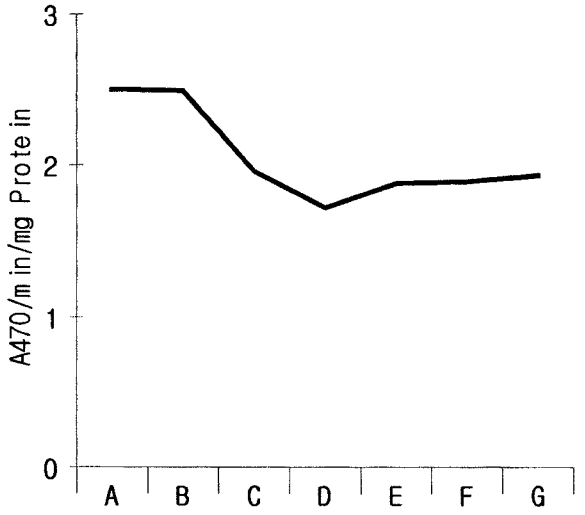


**Fig. 10.** Peroxidase activity in the developmental stage of somatic embryo in *Poncirus trifoliata* RAFIN.
A, Callus; B, 2 weeks culture; C, 4 weeks culture;
D, 6 weeks culture; E, 8 weeks culture;
F, 10 weeks culture; G, 15 weeks culture;

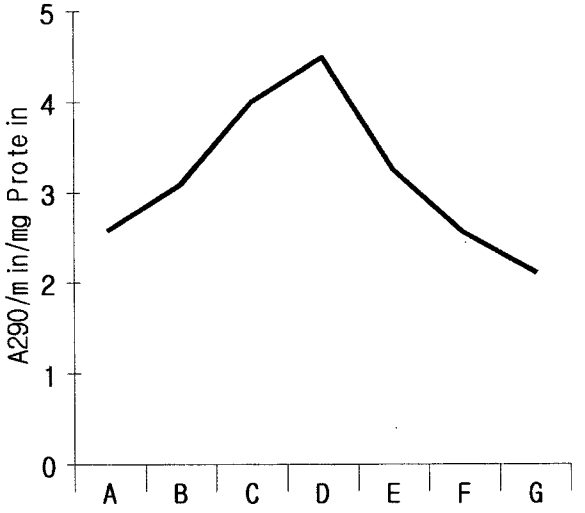


**Fig. 11.** The esterase activity in the developmental stage of somatic embryo in yooja.
A, Callus; B, 2 weeks culture; C, 4 weeks culture; D, 6 weeks culture; E, 8 weeks culture;
F, 10 weeks culture; G, 15 weeks culture.

culture (Fig. 7,8).

In yooja, two marked bands were presented between 40~66kDa. And a lot of bands (12 bands) were appeared at 2~4 weeks culture (Fig. 7). The most remarkable band was observed between 66 and 18.4~14.2kDa at 6~8 weeks culture. An interesting band at 10 weeks culture was observed in about 40kDa and unique band in 31~14.5kDa was appeared at 15 weeks culture.

In the case of trifoliate orange, all protein patterns were showed similar to yooja in all stages. Especially, common bands were showed 2 bands in 21.5~14.5kDa, 3 bands in 45~31kDa and 1 band in 60~45kDa. At 10 weeks culture, most of bands were showed in 66~45kDa and 97.4~ 66kDa. Rich band was presented to 31~21.5kDa in 15 weeks culture.

### The Activity and IEF Patterns of Isozyme in Developmental Stages

***Peroxidase activity*** : The change of peroxidase

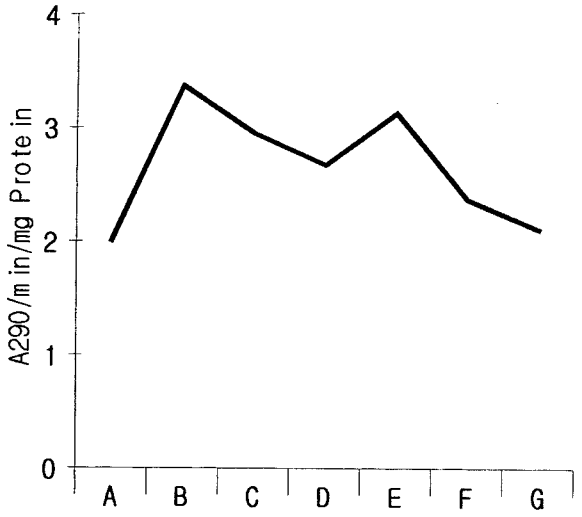


**Fig. 12.** The esterase activity in the developmental stage of somatic embryo in *P. trifoliata* RAFIN.
A, Callus; B, 2 weeks culture; C, 4 weeks culture; D, 6 weeks culture; E, 8 weeks culture;
F, 10 weeks culture; G, 15 weeks culture.

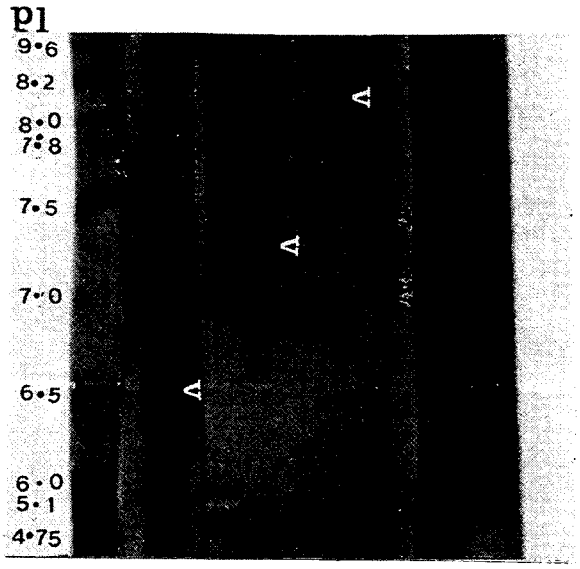


**Fig. 13.** The change of esterase pattern in the various stage of somatic embryo in yooja(*C.junos* SIEB).
A, Callus; B, 2 weeks culture; C, 4 weeks culture; D, 6 weeks culture; E, 8 weeks culture;
F, 10 weeks culture; G, 15 weeks culture.

activity was observed during callus induction and plant regeneration (Fig. 9,10). Peroxidase activity of yooja was greatly increased from callus stage to 8 weeks culture and showed the most prominent activity at mature stage of leaf differentiation (8 weeks culture) but slowly decreased after the stage of plantlet and rapidly decreased at 15 weeks culture (Fig. 9). Also this activity of trifoliate orange was gradually increased between callus to 8 weeks culture and presented the most high activity at 4 weeks culture (Fig. 10).

***Esterase activity*** : Esterase activity of yooja was increased from callus to 6 weeks culture and the most high activity was showed at 6 weeks culture (Fig. 11). However the activity was gradually decreased after 8 weeks culture. Esterase activity of trifoliate orange was increased at 2 weeks culture, but decreased at 4 weeks culture (Fig. 12). Unlike yooja, maximum activity was presented at 8 weeks culture and rapidly decreased after 10 weeks culture. Esterase activity at 15 weeks culture was showed similar to callus stage.

***IEF patterns*** : Fig. 13 and 14 was showed isozyme profile of esterase and peroxidase during plant regeneration. In case of yooja, 10~12 bands were generally appeared at most of stages(Fig. 13). The unique band was found at pI 6.5 of callus stage except for the other stages. Prominent bands at pI 7.7~6.5 were presented at 2 ~4 weeks culture. Significant bands were presented at pI 8.2 (at 8 weeks), pI 7.5~6.5 (at 10 weeks), and pI 7.8~7.5 (at 15 weeks), respectively.

Also trifoliate orange presented 10~12 bands simillar to yooja. two bands at callus stage were identified and unique band was presented at stage of shoot and leaf differentiation. At 6 weeks culture, 2 bands were appeared in pI 7.5~6.5. 2 strong bands were shown in pI 9.6 and pI 7.5~6.0 at 8 weeks culture, respectively (Fig.14). And one unique band in 10~15 weeks culture was presented at pI 7.5~6.0.

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