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Improvement of Black Locust(*Robinia pseudoacacia* L.) Through Tissue Culture.

I. Micropropagation and Somatic Embryogenesis[1]*

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조직배양에 의한 아까시나무(*Robinia pseudoacacia* L.)의 개량

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ABSTRACT

A micropropagation system for black locust (*Robinia pseudoacacia*) was established by using shoots and pin-punctured leaves of *in vitro* germinated seedlings. The greatest number of shoots (an average of 10.5 shoots) was obtained when shoot tips were cultured on MS medium supplemented with 1.0 mg/l BAP and 0.01 mg/l NAA. When pin-punctured leaf explants were cultured on the same medium, mean number of 13.5 shoots were produced. Shoot growth was accelerated by adding 50 mg/l of silver nitrate ($AgNO_3$), an anti-ethylene compound to the culture medium. Each shoot was excised from the mass and transferred onto half strength MS medium for rooting. Zygotic embryos at different developmental stages were cultured on LS medium supplemented with various growth regulators to induce somatic embryos. When cultured on LS medium with 1.0 mg/l 2,4-D, 14.3% of the zygotic embryos induced somatic embryos. Upon transfer onto the basal medium, somatic embryos sporadically converted into plantlets.

Key words : *black locust* (Robinia pseudoacacia), *micropropagation*, *somatic embryogenesis*.

要　約

기내발아된 치묘를 사용하여 아까시나무의 기내대량증식 시스템을 확립하고, 미성숙 접합배로부터 체세포배발생에 미치는 요인을 조사하였다. 기내줄기 대량증식에는 MS기본배지에 1.0mg/l BAP와 0.01mg/l NAA를 첨가해 주었을 때 평균 10.5개의 줄기가 발생되었다. 같은 생장조절물질이 첨가된 MS배지에 침으로 자극한 잎을 치상하였을 때 13.5개의 줄기가 발생하였다. 에틸렌 발생을 억제하는 효과를 가지고있는 $AgNO_3$를 50mg/l 첨가해 주었을 때 줄기기부에 callus발생을 줄여 정상적인 줄기 생장을 유도할 수 있었다. 이들 줄기는 생장조절물질이 첨가되지 않은 1/2MS기본배지에 이식하였을 때 완전한 식물체로 자랐다. LS배지에 2,4-D와 BAP를 단용 또는 혼용한 배지에 발육단계가 다른 여

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러가지 접합배를 이식하여 체세포배를 유도하였다. 그 결과 수정 4주된 접합배를 1.0mg/l 2,4-D를 첨가한 LS 배지에 배양했을 때 14.3%의 체세포배를 얻었다. 이들 체세포배는 매우 낮은 빈도로 완전한 식물체로 발전하였다.

INTRODUCTION

Black locust(*Robinia pseudoacacia* L.) is natively growing from the Appalachian Mountains to central hardwood forest regions of the United States (Hanover, 1992). The usage of this species includes landscaping, erosion control as well as firewood in the world wide. Possibly due to its ability of fixing atmospheric nitrogen, this species shows extraordinary growth performance compared with other woody plants planted in the same area. In Hungary, various genetic improvement programs have been conducted such as timber, feed-stock, and honey production.

Since it was introduced to Korea in 1897, black locust had been planted for the purpose of soil conservation and firewood production until 1970's. Reforested stand area of black locust in Korea was estimated as 325,000 ha at the end of 1991. After Korean Civil War, this species were chosen for artificial reforestation to lessen serious soil erosion of mountain side and planted all over the country. Rapid cycling of nitrogen into the soil improved quickly the soil nutrient status, and also allowed rehabilitation of otherwise non-productive land (Dawson, 1992).

Many genetic improvement programs have been carried out in Korea. However, lack of information on the origin of seeds introduced has hindered genetic improvement works and still remained as a barrier. Thus, we started with desirable genetic traits and aimed to improve the genotype by establishing regeneration system as forest step(Park, 1992).

Large-scale vegetative propagation of selected superior tree is important for reforestation of a genotype having desirable genetic traits. Moreover, *in vitro* regeneration system is readily adaptable for the application of genetic transformation as a part of black locust improvement program(Chalupa, 1992).

Somatic embryogenesis is regarded by many researchers as the method of choice for mass propagation of desirable genotypes due to a number of advantages over both conventional clonal propagation method(e.g. rooted cuttings) and *in vitro* propagation systems(Merkle, 1992).

In this paper, we report a reliable system for micropropagation as well as somatic embryogenesis from black locust.

MATERIALS AND METHODS

Micropropagation

Seeds of black locust were kindly provided from Forest Research Institute in Kyungju, Kyungsangpuk-do, Korea. Seeds were surface-sterilized with 70%(v/v) ethanol for 1 min and 1%(v/v) sodium hypochlorite for 1 hr, and then 1%(v/v) hydrogen peroxide for 1 hr. All media used were adjusted to pH 5.8 prior to autoclaving for 15 min at 121℃. Cultures were maintained at constant temperature of 25℃ with 2,000 to 3,000 lux of illumination with cool white fluorescent lamp for 16 hr photoperiod. Three-week-old *in vitro* germinated seedlings were used as an explant for micropropagation.

Shoots were cultured on MS(Murashige and Skoog, 1962) basal medium supplemented with various concentrations of BAP. To confirm shoot proliferation capacity, shoot explants were tested using various combinations of auxins(NAA, 2,4-D) and cytokinin(BAP). Leaf explants were punctured 10 times with a sharp pin to stimulate shoot induction (for morphogenic response), and abaxial side of leaf was placed on MS medium supplemented with 1.0 mg/l BAP combined with NAA and 2.4-D. Number of shoots induced was recorded after 8 weeks, but cultures were routinely maintained for a period of 10 weeks. To determine optimal level of anti-ethylene compound(silver nitrate), shoots were placed on multiplication medium containing 0.0, 1.0, 5.0, 10.0, 30.0, 50.0, 70.0, and 100.0 mg/l $AgNO_3$. To increase both shoot growth and rooting rate, each shoot was subcultured onto various culture media :

ACM(Ahuja, 1983), B_5(Gamborg *et al.*, 1968), GD (Gresshoff and Doy, 1972), LS(Linsmaier and Skoog, 1965), WPM(Lloyd and McCown, 1981), MS and modified MS(1/4MS, 1/2MS, 3/2MS and 2 MS) medium.

Rooted shoots were transplanted to pots containing artificial soil mixture(peat : vermiculite=1 : 1) and kept in a growth chamber with 25℃ and 80% relative humidity.

Somatic embryogenesis

Immature zygotic embryos were taken from the pods of black locust growing at campus of Kyungpook National University in Taegu. Developing fruits were collected from 30 sample trees at three day intervals. Pods were collected from 25 May(at the time of flowering) to 26 July(at the time of maturating pods), 1993. Pods were surface-sterilized with 70% ethanol for 20 sec, 2% sodium hypochlorite for 30 min. and rinsed 3 times with sterilized distilled water.

After disinfestation, pods were cut lengthwise and individual seeds were excised to isolate embryos, and then cultured on LS medium with or without plant growth regulators. Cultures were maintained at 25℃ culture room under dark conditions. The results of embryos induction were determinded by counting the number of embryos induced after 6 weeks of initial culture.

RESULTS AND DISCUSSION

Micropropagation

Multiplication was occurred when shoots were cultured on MS medium supplemented with 0.01 to 5.0 mg/l cytokinins. Of all the growth regulators tested, BAP yielded the highest number of shoots from the explants. Shoot multiplication was best when shoots were cultured on MS medium with 1.0 mg/l BAP(Fig. 1).

The NAA supplemented medium promoted shoot multiplication. A mean number of 10.5 shoots was obtained on MS medium containing 1.0 mg/l BAP and 0.01 mg/l NAA(Fig. 2). Chalupa(1983) reported that suitable medium for axillary bud culture of black locust was modified suitable medium for axillary bud MS medium supplemented with 0.4 to 0.6 mg/l BAP and 0.05 mg/l IBA. Barghchi(1987) also reported similar results using the same species with juvenile tissues isolated from root cuttings.

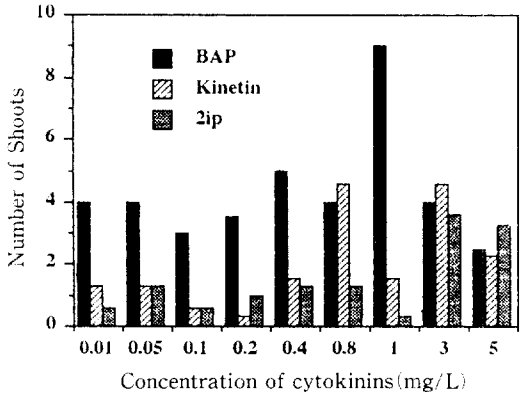

Fig. 1. Shoot multilplication from axillary bud on MS medium with various cytokinins.

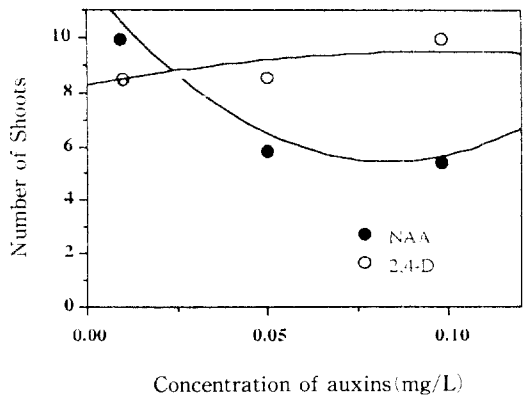

Fig. 2. The number of shoots initialted from axillary bud on MS medium with 1.0 mg/L BAP combined NAA and 2,4-D.

When pin-punctured leaf explants were cultured on MS medium containing 1.0 mg/l BAP and 0.01 mg/l NAA, mean 13.5 shoots per leaf explant were produced(Fig. 3). Multiple shoots were mostly induced from callus formed on pin-punctured area of abaxial side or sparsely from cut ends of petiole. Shoot number induced from pin-punctured leaf culture was higher than that of axillary bud cultures. Shoot number induced from abaxial side of pin-punctured leaf decreased with increase of auxin concentrations. Similar results were previously re-

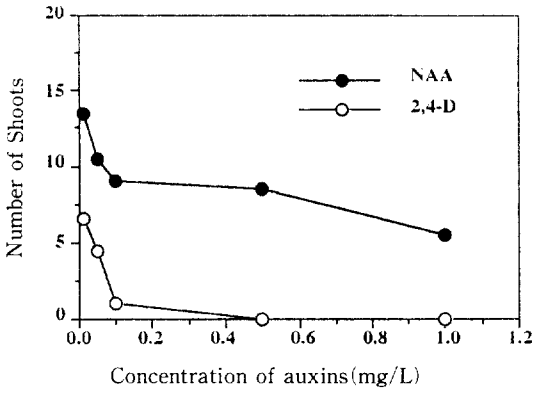

Fig. 3. Shoot multiplication in abaxial side culture of pin-punctured leaf on MS medium with 1.0 mg/L BAP combined with NAA and 2, 4-D.

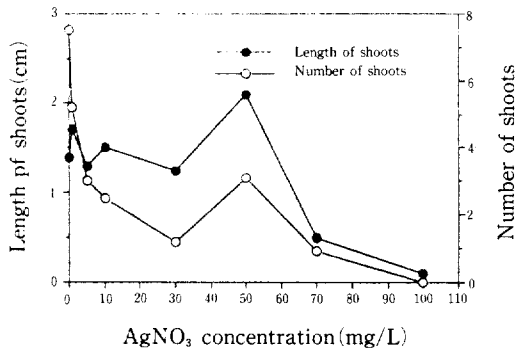

Fig. 4. Effects of $AgNO_3$ concentrations on the length of shoots and the number of multiple shoots on MS medium containing 1.0 mg/L BAP, 0.01 mg/L NAA after 6 weeks of culture

ported using pin-punctured leaf culture of *Populus* spp. in which relatively large number of shoods were induced via somatic embryogenesis or organogenesis followed by callusing from the cultured tissues(Park and Son, 1988).

When shoot was cultured on proliferation medium containing various concentrations of $AgNO_3$, formation of shoot mass and its elongation occurred after 2 to 5 weeks of inoculation. Number of shoots proliferated was high on MS medium containing low concentrations of $AgNO_3$(Fig. 4). There seems to be additive relationships between the level of $AgNO_3$ and the number of shoot induced. Optimum concentration of $AgNO_3$ for shoot induction seems to be 50 mg/l. Obstacle to shoot and root induction of given

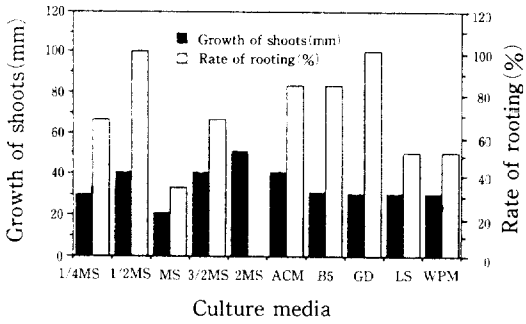

Fig. 5. Effects of culture media on the growth of shoots and *in vitro* rooting after 4 weeks in culture.

species is callusing at the basal part of shoot. It seems that callus at the basal part of shoot hinders rooting and shoot growth, because it prevents explants from uptaking nutrient.

Although the effect of accumulated ethylene in tissue culture vessels is not well known, the addition of $AgNO_3$ in the medium seems to be effective for inhibition of callus formation on the shoot base and promotion of multiple shoot production. Chi *et al.* (1989) reported that $AgNO_3$ was effective for shoot and root organogenesis from recalcitrant *Brassica* genotypes.

After multiplication, the shoots proliferated on the medium containing 50 mg/l $AgNO_3$ were transferred onto several rooting media. Rooting percentage was higher on the low salt media such as 1/4MS, 1/2MS, GD, B_5, and ACM. When the shoots were cultured on the medium containing high salt, they induced callus and proliferated. High rooting percentage was also obtained on half strength MS medium(Fig. 5). Decreasing inorganics in medium at early rooting stage of papaya, *Dracalna fragans* and *Targetes patula* as well as other woody plants have been recommended(Bonga, 1982 ; Drew, 1987 ; Usha and Martin, 1991 ; Vinterhalter and Vinterhalter, 1992). Based on this observation, we have chosen 1/2MS and GD media for rooting medium in our experiment. Successful transplantation into soil mixture and further growth were achieved with most of the plants transplanted.

Somatic embryogenesis

When immature embryos cultured on LS medium

with 2, 4-D alone, or combination with BAP, somatic embryos were induced. After 2 weeks of culture, first proembryoid was observed on LS medium with 2, 4-D. Somatic embryo formation from immature embryo was observed with the frequency of 14.3% on LS medium with 1.0 mg/l 2.4-D at estimated 4 weeks after anthesis. Table 1 shows the effect of growth regulators on somatic embryogenesis. Clumps of somatic embryos at different developmental stages transferred to LS medium and converted to plantlets within 8 weeks. The inductions of somatic embryos depended on the conditions of donor explant and seed maturation(Data not shown). This result suggested that somatic embryogenesis of black locust may depend on anthesis time of zygotic embryo. Upon transferring onto the basal medium, the somatic embryos were converted into plantlets. Although induction of somatic embryos from black locust is successful, the maturation and germination of the somatic embryos are still problematic.

Merkle(1992) reported that 3 day culture of seeds collected 2 weeks after anthesis and on MS medium supplemented with 10 mg/l 2, 4-D and 0.25 mg/l BAP before transferring to basal MS medium was effective. Somatic embryos were produced directly from the radicles of zygotic embryos and new embryos were produced continually via repetitive embryogenesis.

Table 1. Effects of growth regulators on somatic embryogenesis of *R. pseudoacacia*.

Growth regulators (mg/l)		Frequency of somatic embryo formation (%)
2, 4-D 0.1		-*
0.5		10.0±0.21**
1.0		14.3±1.34
2.0		7.7±1.0
4.0		-
10.0		-
20.0		-
30.0		-
2, 4-D 0.1	BAP 0.1	-
0.5	0.1	11.1±2.15
1.0	0.1	-
2.0	0.1	-
4.0	0.1	-

* No response

** The values represent mean±standard deviation

Various treatments of growth regulators have been applied to induce somatic embryogenesis in woody legumes. Embryogenic callus cultures of *Cercis canadensis* and *C. lutea* were routinely obtained by using of IAA or 2, 4-D(Trigiano *et al.*, 1988; Geneve and Kester 1990). Also, somatic embryogenesis by tissue cultures of the *Albizzia* species was induced using BAP(Tomer and Gupta, 1986, 1988) or without growth regulators(Gharyal and Maheshwari, 1981). It is still difficult to induce somatic embryos from woody legumes although some were successful by using different culture media and different growth regulators. Problems related to abnormality and low germination frequency of somatic embryos of black locust are also to be solved for further use of this system. However, the results reported in this paper will be helpful for genetic improvement of this species and can be applied to other important tree species.

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Fig. 6. Multiplication and somatic embryogenesis of black locust. A. Effects of $AgNO_3$ concentrations on MS medium containing 1.0 mg/l BAP, and 0.01 mg/l NAA after 6 weeks of culture. A-1. without $AgNO_3$, A-2. 5.0 mg/l $AgNO_3$, A-3. 50.0 mg/l $AgNO_3$, B. Rooted plants on various medium after 4 weeks in culture. B-1. 3/2 MS, B-2. GD, B-3. 1/2 MS, C. Habituated plant D. Multiple shoots from pin-punctured leaf on MS medium with 1.0 mg/l BAP and 0.01 mg/l NAA. E. Somatic embryos formed on immature zygotic embryo.

critical reading of the manuscript.

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