식물조직배양학회지 제22권 제3호
Korean J. Plant Tissue Culture
Vol. 22, No. 3, 127~130(1995)

Micropropagation of Superior Variety of Japanese Pepper Tree (*Zanthoxylum piperitum* Dc.)

Sung Ho SON*, Seong Doo HUR[1], Jung-Hee KIM[2], Yun Hee LEE, Mee Hee KIM, Jin Seon PARK, Yong Wook LEE, Heung Kyu MOON, Yang YOUN, and Suk Koo LEE

Division of Biotechnology, [1]Division of Nut Tree, Forest Genetics Research Instiute, Forestry Administration, Suwon, 441-350: and [2]School of Forestry, Michigan Technological University, 1400 Townesend Drive, Houghton, MI 49931-1295, USA. *Corresponding author.

수형목 민초피나무의 기내 대량 증식

손성호* · 허성두[1] · 김정희[2] · 이윤희 · 김미희 · 박진선 · 이용욱 · 문흥규 · 윤 양 · 이석구

임목육종연구소 생물공학과, [1]유실수과, [2]Michigan Technological university, USA

Japanese pepper (*Zanthoxylum piperitum* Dc.) tree of selected genotype was propagated by a two-step method. Among the media tested, BTM promoted shoot height growth and DKW revealed as superior to micro-canopy increment. Multiple shoot formation was greatest when the single shoots were subcultured on medium containing 0.89 μM BA alone. The numbers of survival shoots could be markedly increased by acclimatization of the multiplied shoots itself in plastic Petri dish (punctured with pin on the top) for two weeks and subsequently transplanting each shoots onto peat plug system. After transplanting the micropropagules onto pots, prickleless characteristics seem to be transmitted to all plants produced from selected genotype.

Key words: in vitro, selected genotype, two-step propagation, woody plant

Japanese pepper (*Zanthoxylum piperitum* Dc.) belongs to the family Rutaceae and is a large shrub or small tree. Although a few species of Zanthoxylum are distributed from northern China to the southern parts of Japan, Korea is considered to be in the center of its natural range. In the orient, their aromatic young leaves, male flowers, fresh seed, ripened seed carpels, bark, and wood of the tree are widely used for a pungent source of spice (Abe et al., 1974: Yasuda et al., 1982). They are also used for medicinal purposes both in home remedies and in the pharmaceutical industry. In Korea, 200 metric tons of wild young leaves and seeds are annually collected for local consumption and export to Japan. Recently, those natural resources and the products derived from them have become less available, mainly due to careless collection and heavy competition in unmanaged forests. Nevertheless, consumption and demand are rapidly increasing. Moreover, prickles located on its bark make for proper harvest difficult. In order to meet the increasing demand, it will be necessary to breed trees having characteristics of rapid growth, larger fruit/clusters, fewer or no prickles, and be prolific. Japanese pepper can be propagated better by using mature stem cuttings than by using juvenile ones, and readily rooted in soil. Although this tree can be vegetatively propagated by conventional methods (i.e., cuttings or graftings), this approach has its own disadvantages. These include limited availability of a superior genotypes, grafting incompatibilities, and high labor requirement. In this study, we describe a rapid and reliable system for the commercial-scale micropropagation of superior genotypes.

MATERIALS AND METHODS

Selection of Superior Varieties of Japanese Pepper Tree

Since this species is generally founded in the mixed forests or as scattered individuals, candidate trees were selected from 23 geographically distinct regions in South Korea. A total of 102 individuals were selected as candidate trees from their habitats. The parameter for selection were prickleless, fruit size, grain weight, and prolific characteristics.

Plant Materials

Stem node segments having one or two axillary buds were collected from actively growing greenish branches of the selected superior trees (Cheonju No.2). After removal of all leaves, branches were disinfected using a previously reported method (Park and Son, 1986). In vitro shoot cultures were obtained by using the bud culture system of Son and Hall (1990). To obtain a sufficient number of plants having similar physiological conditions, axillary bud sprouts were excised, then 5 bi- or tri-nodal shoots were cultured in baby food jars (5.5 × 11.0 cm) containing 20 ml of woody plant medium (WPM) supplemented with 4.43 μM BA. After 4 subcultures, with a 6-week interval on the same medium, each shoot culture isolated from multiplied axillary branches were maintained on WPM (Lloyd and McCown, 1981) without plant growth regulators (PGRs) for more than 6 weeks to eliminate the dosage affect of previous culture media. Plants of 4 to 6 cm in height with fully expanded leaves were used as in vitro source materials.

The Effect of Media Types for Shoot Growth

Plant tissue culture media, BTM (Chalupa, 1984), MS (Murashige and Skoog, 1962), 1/2MS, WPM, GD (Gresshoff and Doy, 1972), and DKW (Driver and Kuniyuki, 1984) were tested for their effects on shoot height and micro-canopy increments. Each shoot from in vitro sources was cultured in baby food jars containing 20 ml of the medium. The pH of the medium was adjusted to 5.7 before addition of 0.6% (w/v) Difco Bacto agar and autoclaved at 1.1 kg cm^{-2} (121° C) for 15 min. The vessels were sealed with a transparent cap and incubated in a culture room at 26±1°C with 60± 10% external relative humidity, 16 h photoperiod, and a photosynthetically active photon flux rate of 40-60 μE^{-2} s^{-1} from cool white fluorescent tubes. Then the vessels were randomly placed on the culture shelves to minimize micro-environmental effects. Each treatment consisted of 5 replicates and the experiment was repeated three times at 3-month intervals. Increments for the shoot height and micro-canopy were measured after 6 weeks of culture. The length of micro-canopy was determined by averaging the size of the biggest and smallest diameter of branch (canopy) in vitro.

Shoot Proliferation and Acclimatization

To test the ability for the shoot multiplication, single shoots from aseptic cultures was removed their apices and cultured on medium containing various types of cytokinins and auxins alone or in combinations ranging from 0 to 50 μ M. Culture media were prepared automatically by using of DM 150 Petri dish filter connected with Medium autopreparator SH 105 (both products of Jouan Co., France) and gave a 12% slope. Five shoot cultures removed their apex were cultured for six weeks on the deep side of the media tested. At this time, culture vessels were placed on the culture shelves horizontally. When multiple shoots were induced, one or two dominantly growing shoots were removed from the multiplied shoot masses and subcultured on same media without PGR. The culture vessels were, then placed upright (longitudinally) to elongate the proliferated shoots. The culture vessels were punctured with pin on the top and placed in the greenhouse. Proliferated and elongated shoot cultures on plastic Petri dishes were placed under the greenhouse conditions where a solar screen was installed to give 30% shade. After 2 weeks of acclimatization in plastic Petri dishes, all plants were excised at their base and directly transplanted onto Poly Terra Peat Plugs (M40045, Techniculture Co., Salinas, CA) to achieve ex vitro rooting. Four weeks after transplantation onto the peat plugs, fertilizer (20-10-20) was applied weekly during winter, spring, fall, and twice weekly during the summer.

RESULTS AND DISCUSSION

Selected genotype of pepper trees showed mean age of 11 years, 2.5 m in height, and had a basal area of 0.67 m^2. Trees selected from Shamchuck and Khoisan produced excellent fruit on short stalks with a dark green color. Total of 11 individuals including Khoisan No.1 and Seungju No.7 were selected as being the most prolific. They showed more than 20 fruit clusters per twig with the largest cluster containing 8 individual fruits and found 28 grains per cluster. Grain size was also greater in selected individuals. For example, the average number of grain was 9,524, whereas individuals with the largest fruit showed 8,717 grains per liter.

Table 1. Effect of the media tested for shoot height and micro-canopy increment in vitro of Japanese pepper tree (*Zanthoxylum piperitum* Dc.).

Type of media	BTM	MS	1/2MS	WPM	GD	DKW
Shoot height increment(cm)	1.37a	0.83b	0.61d	0.78bc	0.70c	0.77bc
Micro-canopy increment(cm)[a, b]	1.24e	3.69b	2.67d	3.25bc	3.10c	4.25a

[a] Numerical values were collected after 4 weeks of culture from 5 replicates each in 3 experiments.
[b] Values followed by different letters are significantly different at P=0.05 following Duncan's multiple range test.

Fruit grain weight was 55.3 g/1,000 grains in average. Among the candidate trees, most notably, Shamchuk No.5 and Cheonju No. 2 were nearly prickleless and large leaf areas. Cheonju No. 2 was finally selected on the basis of fruit traits and prickle state. Its main characteristics were 25.9 grains (1.47 g fresh weight) per cluster and 8,817 grains per liter. These features are 1.6, 1.5, and 2.1 times greater than normal, respectively.

The earliest visible response, within 7 days of culture, was green in over 99% of the single shoot derived from in vitro stock materials. Maintained for more than 2 weeks, however, some of the shoot cultures turned pale greenish to red. This phenomenon was possibly attributable to the nutritional insuitability. When different types of culture media were tested, a few of the media eliminated the obsolescence mentioned above and represented rapid growth. Because F test on each treatment in the ANOVA reveals highly significant differences at $\alpha = 0.05$, the response of shoot growth on different types of media was analyzed by Duncan's multiple range test. Tables 1 summarize the growth performance of shoot cultures in tested media. Although shoot height increment using BTM appeared to be slightly effective, based on its mean value, micro-canopy increment was greatly increased by the incorporation of DKW (Table 1). Further experiments on shoot multiplication were therefore conducted with DKW as basal medium. Calculation of micro-canopy was conducted by the principles of field application. Although this kind of measurement is an unusual parameter for evaluating in vitro growth of other species, it seems to be a highly effective standard for growth index of tissue cultured Japanese pepper because of its special growing habit in vitro environment.

In general, multiple shoot formation was greatest at low concentration (ca. 0.9 μM) of BA when used singly (Figure 1). Both BA alone and BA with IBA treatments resulted in leaves that were pale greenish to light yellow on their margins. When other types of cytokinins (2iP and kinetin) and auxins (IAA and NAA) were used, the total number of multiple shoots were greatly decreased (data not shown). In some cases, autumn coloration occurred on the shoot cultures when maintained on media containing high levels of PGR, except zeatin. There was an increase in the number of multiple shoot buds when these proliferated shoot masses were transferred to the same fresh medium without PGR. By the association of zeatin, most of the multiplied shoots could maintain healthy green color. Although zeatin seems to be suitable, due to its expensive cost, detailed experiments using the growth substance was excluded in this study. The rooting ability in vitro of this genotype varied depending on the agar and IBA concentration. When 1.0% of agar was associated in culture medium, rooting frequency was below than 5%. While, the multiple shoot cultures produced on Perti dishes were acclimatized in itself (by puncturing on the top and placing under the greenhouse conditions) and directly transplanting each shoot onto peat plug system, almost 100% of shoots produced root ex vitro. We are now able to propagate by a two-step method on several clones, especially Cheonju No. 2

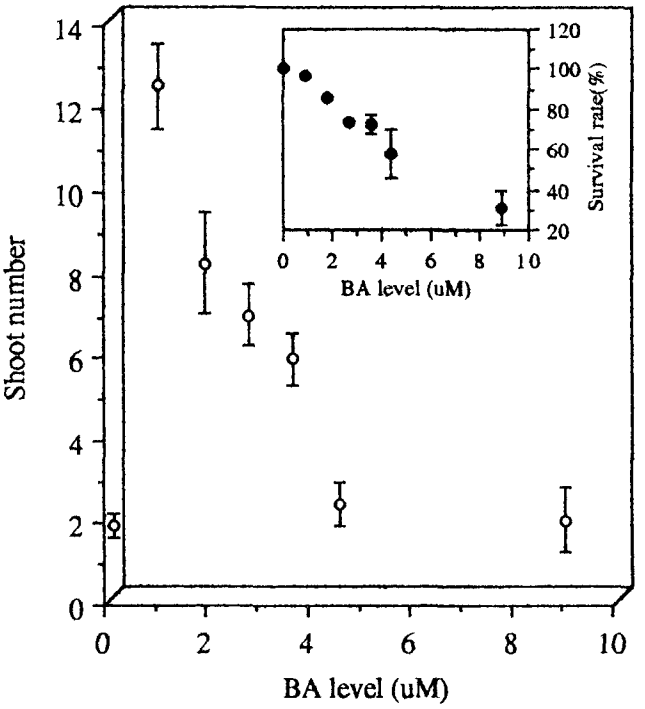

Figure 1. Effect of BA on shoot multiplication of in vitro grown Japanese pepper tree (*Zanthoxylum piperitum* Dc.). Each point represents the mean of 15 replications. Vertical bars denote standard deviation.

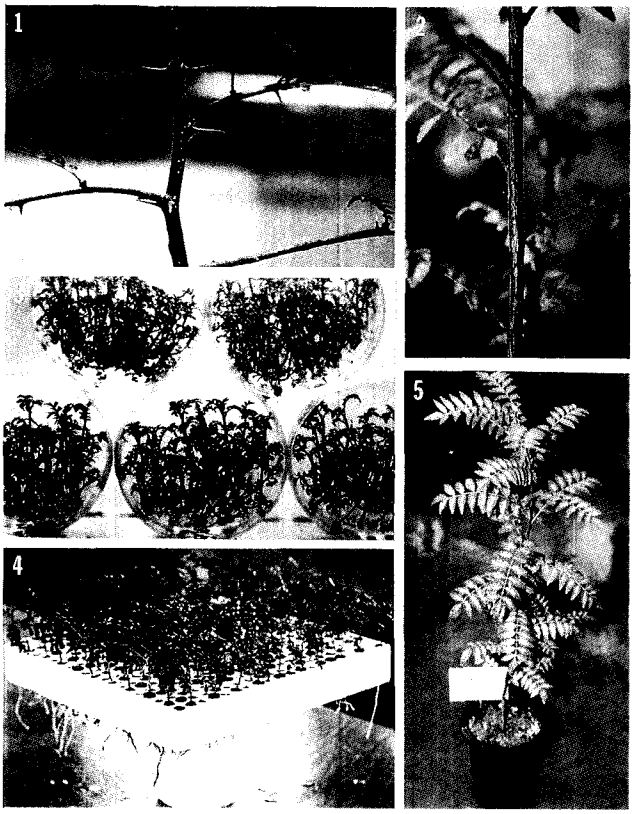

Figure 2. Micropropagation of Japanese pepper (*Zanthoxylum piperitum* Dc.) tree: (1) typical features of japanese pepper with prickles, (2) selected genotype without prickle, (3) shoot multiplication, (4) ex vitro rooting with peat plug system, and (5) an *in vitro* cultured plantlets after six months of transplanting into pot.

with successful transmission of prickleless characteristic of the genotype (Figure 2). Based on the results, approximately 7,000 plantlets could be produced every 10 weeks (with 100 Petri dishes with 5 shoot cultures) and 35,000 plantlets annually. This opens up the possibility of commercial scale micropropagation of selected pepper tree.

적 요

생산성이 높고 가시가 없는 초피나무의 2단계 대량증식이 가능하였다. 기내 줄기의 생장에는 대체로 DKW 배지가 좋은 반응을 보였으며, 수평으로 120°각도를 준 플라스틱 Petri dish에서 다경 줄기를 유도할 때에는 BAP 0.89 μM이 적당한 것으로 나타났다. 다경 줄기를 유도한 후, 수직으로 세운 Petri dish의 윗면에 5-7개의 구멍을 낸 다음, 30% 정도의 빛만 투과할 수 있는 조건의 온실에서 환경순화를 시켰다. 이들 다경 줄기의 기저부를 제거한 다음 각각의 줄기를 피트 플러그에 이식한 결과, 100%의 발근율을 얻었다. 폿트에서 약 6개월 가량 유지하였을 때에도 선발목에서와 같이 줄기에 가시가 형성되지 않았으며, 향후 생산성에 대한 검정이 이루어지면, 조직배양에 의한 실용화가 가능할 것으로 생각된다.

ACKNOWLEDGMENTS - This research has been partially supported by a special grant from the Ministry of Science and Technology. We are grateful to Dr. Richard Meilan for his critical reading of this manuscript.

REFERENCES

Abe F, Yahara S, Kubo K, Nonaka G, Okabe H, Nishioka I (1974) Studies on *Zanthoxylum* spp. II. Constituents of the bark of *Zanthoxylum piperitum* Dc. Chem Pharm Bull **22**: 2650-2655

Chalupa V (1984) In vitro propagation of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). Biol Plant **26**: 374-377

Driver JA, Kuniyuki AH (1984) In vitro propagation of Paradox walnut root stock. HortScience **19**: 507-509

Gresshoff PM, Doy CH (1972) Development and differentiation of haploid *Lycoperscion esculentum* (tomato). Planta **107**: 161-170

Lloyd GB, McCown BH (1981) Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip culture. Prop Int Plant Proc Soc **30**: 421-427

Mushige T, Skoog F (1962) Revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol Plant **15**: 473-479

Park YG, Son SH (1986) Factors affecting the isolation of mesophyll protoplasts from *Populus euramericana* cv. I-214. J Korea For Soc **74**: 29-36

Son SH, Hall RB (1990) Multiple shoot regeneration from root organ cultures of *Populus alba* X *P. grandidentata*. Plant Cell Tissue Organ Culture **20**: 53-57

Yasuda I, Takeyama K, Itokawa H (1982) Distribution of unsaturated aliphatic acids in Japanese *Zanthoxylum* species. Phytochemistry **21**: 1295-1298

(Received March 20, 1995)