Short Communication

Somatic Embryogenesis from the Leaf Tissues of Continuously Subcultured Shoots in Japanese Persimmon (*Diospyros kaki* THUNB.)

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Indirect somatic embryogenesis occurred in Japanese persimmon 'Fuyu' leaf segments which were cut from shoots one year plus four or five weeks after the beginning of subculturing on Murashige and Skoog's medium which contained half strength nitrogen (1/2 NMS) supplemented with 10^{-5} M zeatin. Embryo formation was increased by the addition of 10^{-6} M BAP and 10^{-5} M NAA to the same 1/2 NMS medium in which the shoots had been cultured. Leaf segments planted with their obverse sides in contact with the medium showed a higher percentage of embryo formation than those planted with their reverse sides in contact.

KEY WORDS : *Diospyros kaki* THUNB., Japanese persimmon, somatic embryogenesis, leaf tissue culture.

Introduction

Recently *in vitro* improvement by selection of cells, protoplast fusion and bioengineering has been undertaken in various plants. Organogenesis or embryogenesis has, therefore, been indispensable to *in vitro* improvement. However, Japanese persimmon have little organogenetic potential (YOKOYAMA 1981) and until now have shown no somatic embryogenesis. The *in vitro* improvement of Japanese persimmon has, therefore, made little progress; only *in vitro* selection of plantlets and micropropagation has been undertaken (FUKUI *et al.* 1987, SUGIURA *et al.* 1986). This report describes somatic embryogenesis from the leaves of the 'Fuyu' persimmon.

Materials and Methods

Aseptic shoot cultures were initiated from the shoot tips (1 mm) of Japanese persimmon 'Fuyu' using Murashige and Skoog's medium (MURASHIGE *et al.* 1962) containing half strength nitrogen (1/2 NMS) with 3% sucrose and 0.7% agar supplemented with 6-(4-Hydroxy-3-methyl-trans-2-butenylamino) purine (zeatin) at 10^{-5} M (FUKUI *et al.* 1988). Successive shoots were obtained by cutting into single node segments elongated shoots originally cultured from shoot tips and subcultured in the same medium as that of the original shoot tips over a period of one year. Leaf segments 5×7 mm square were cut from the subcultured shoots and placed with their reverse sides in contact with the 1/2 NMS medium to which was added 6-benzylaminopurine (BAP) and Naphthaleneacetic acid (NAA) in various combinations and concentrations. Cultures were maintained at 25°C for 16 hr light periods at about 3000 lux. These procedures were also performed with

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the obverse side of segments in contact with the medium.

Results and Discussion

Somatic embryos were induced from calli formed at the cut ends of leaf segments. These embryogenic calli were white or light green, nodular and compact (Fig. 1 A). Somatic embryos with the same color as calli were distinguished calli by hypocotyl with epidermal system and came off calli easily. All embryos formed had extraordinary shaped

cotyledons which were trumpet-shaped and callused cotyledon (Fig. 1 B and C).

Somatic embryogenesis occurred in the leaves in the fourth or fifth week after the beginning of subculturing but not at all in the sixth week, although the sixth week marked maximum formation of calli (Table 1). The greater capability of embryogenesis in the younger leaf explants, therefore, may be related to their age.

Embryos were observed on leaf segments cultured in media containing either 10^{-5} M or 10^{-6} M BAP and 10^{-5} M NAA, while a high concentration of NAA (10^{-4} M) inhibited embryogenesis with either BAP concentration for any length of time (Table 1). To ascertain the optimal combination of cytokinin and auxin concentration for embryogenesis, leaf segments were cultured on media supplemented with various combinations of higher or lower concentrations of 1.0×10^{-6} M BAP and 1.0×10^{-5} M NAA (Table 2). Callus formation occurred at about the same rate for all combinations except that of 3.3×10^{-7} M BAP and various concentrations of NAA (Table 2). Embryogenesis was observed in nearly forty percent of the leaf segments cultured in a medium with 1.0×10^{-6} or 3.3×10^{-6} M BAP and 1.0×10^{-5} or 3.3×10^{-5} M NAA. However, less embryogenesis occurred with 10^{-5} M BAP than with 10^{-6} M (Table 3). Favorable combinations of BAP and NAA, therefore,

Fig. 1. Embryogenic calli (A) and somatic embryos with trumpet-shaped cotyledon (B) and callused cotyledon (C) were induced from leaf segments. Arrow shows the somatic embryo.

were 1.0×10^{-6} to 3.3×10^{-6} M BAP with 1.0×10^{-5} to 3.3×10^{-5} N NAA.

Which side of the leaf segment was in contact with the medium made an obvious difference: the percentage of embryo formation was only 35.7% in the case of reverse side contact but 66.7% for obverse side contact (Table 3).

Somatic embryogenesis from leaf segments of subcultured shoots of 'Fuyu' occurred co-

Table 1. Somatic embryos observed on leaf segments cut at different times from shoots already cultured for one year. Leaf segments cultured in various combinations of NAA and BAP.

Length of time before cutting	Hormones in medium (M)		callus formation (%)	somatic embryogenesis
	BAP	NAA		
four weeks	10^{-5}	10^{-4}	21.4 (3/14)	—
	10^{-6}	10^{-4}	42.9 (9/21)	—
	10^{-5}	10^{-5}	76.2 (16/21)	+
	10^{-6}	10^{-5}	71.4 (15/21)	+
five weeks	10^{-5}	10^{-4}	90.5 (19/21)	—
	10^{-6}	10^{-4}	33.3 (7/21)	—
	10^{-5}	10^{-5}	66.7 (14/21)	+
	10^{-6}	10^{-5}	90.5 (19/21)	+
six weeks	10^{-5}	10^{-4}	90.5 (19/21)	—
	10^{-6}	10^{-4}	95.2 (20/21)	—
	10^{-5}	10^{-5}	71.4 (15/21)	—
	10^{-6}	10^{-5}	100.0 (21/21)	—

Table 2. Effect of various combinations and concentrations of NAA and BAP on somatic embryogenesis and callus formation.

Hormones in medium (M)		callus formation (%)	somatic embryogenesis (%)
BAP	NAA		
3.3×10^{-7}	3.3×10^{-6}	35.7 (5/14)	0 (0/14)
1.0×10^{-6}	3.3×10^{-6}	71.4 (15/21)	0 (0/21)
3.3×10^{-6}	3.3×10^{-6}	86.7 (13/15)	26.7 (4/15)
3.3×10^{-7}	1.0×10^{-5}	19.0 (4/21)	9.5 (2/21)
1.0×10^{-6}	1.0×10^{-5}	85.7 (12/14)	35.7 (5/14)
3.3×10^{-6}	1.0×10^{-5}	100.0 (21/21)	38.1 (8/21)
3.3×10^{-7}	3.3×10^{-5}	9.5 (2/21)	4.8 (1/21)
1.0×10^{-6}	3.3×10^{-5}	95.2 (20/21)	38.1 (8/21)
3.3×10^{-6}	3.3×10^{-5}	100.0 (14/14)	35.7 (5/14)

Table 3. Somatic embryogenesis and callus formation when leaf segments were placed on media with reverse or obverse sides of leaves in contact with the medium.

Hormones in medium (M)		reverse or obverse	callus formation (%)	somatic embryogenesis (%)
BAP	NAA			
10^{-6}	10^{-5}	reverse	85.7 (12/14)	35.7 (5/14)
10^{-6}	10^{-5}	obverse	100.0 (21/21)	66.7 (14/21)
10^{-5}	10^{-5}	reverse	100.0 (21/21)	4.8 (1/21)
10^{-5}	10^{-5}	obverse	100.0 (21/21)	0.0 (0/21)

tinuously under the above conditions. TULECKE (1987) indicated that the embryogenic potential of cells frequently depends on the conditions under which the source plant was grown, and DURZAN (1984) refers to the relation between the embryogenic potential and the juvenility of source plant tissues, particularly in woody perennial species. Despite the low embryonic potential in woody perennial species, especially Japanese persimmon, in this experiment high embryonic potential prevailed. This may have resulted from the leaf explants being cut from continuously subcultured shoots.

No embryos developed into plantlets. SONDAHL *et al.* (1977) observed somatic embryogenesis from mature coffee leaf segments and reported that these embryos were induced by transplanting leaf segments from a medium containing high concentrations of cytokinin (2×10^{-5} M) and auxin (5×10^{-6} M) to one containing low concentrations (2.5×10^{-6} M and 2.5×10^{-7} M respectively), and that thereafter the embryos developed into normal plantlets. Embryo development into plantlets may be related to transplantation into a medium with a low concentration of plant growth regulators.

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継代培養中のカキのシュートの葉からの体細胞胚形成

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窒素成分を 1/2 にした MS 培地 (1/2 NMS) に zeatin 10^{-5} M を添加した培地で茎頂培養を行い, その後伸長したシュートを節毎に切り取り, 同じ組成の培地で継代培養して約 1 年間維持されている“富有”のシュートから採取した葉を供試材料とした. 1/2 NMS 培地に BAP 1.0×10^{-6} M と NAA 1.0×10^{-5} M を組み合わせて添加した培地で高い体細胞胚形成率を示した. 体細胞胚形成は, 継代培養開始 4 週間後と 5 週間後の葉に限られ, 体細胞胚形成能と葉齢との間には密接な関係があった. 置床方法では, 葉の表を培地面に接した場合 66.7% の不定胚形成率を示し, 裏を培地面に接した場合の 1.5 倍となった. embryogenic なカルスは薄い緑色または白色で, 微細な粒状の突起を呈していた. 不定胚は表皮系を持った胚軸部と子葉を持ち, カルスから容易に分離できることから, embryogenic なカルスと区別することができた. 形成された不定胚はラッパ状の異常な子葉を持ち, 中にはその子葉部が再びカルス化するものも観察された, これらの不定胚は植物体まで生育させることができなかった.