J. Plant Biotechnology(1999) *Vol. 1(2). pp.91~95*

Journal of Plant Biotechnology

Tissue Culture of Stone Fruit Plants Basis for Their Genetic Engineering

Marta Csányi, Anita Wittner, Ágnes Nagy, Ildiko Balla, Judit Vértessy[1], Laszlo Palkovics, Ervin Balázs*

Agricultural Biotechnology Center H-2100 Gödöllö, Szent-Györgyi Albert Street 4, Hungary; [1]Ornamental and Fruit Crop Research Institute Budapest H-1223 Park Street 2, Hungary.

Key words: apricot, plum, regeneration, TDZ

Abstract

Genetic engineering of stone fruit species like apricot, plum, peach and cherry are hampered by the inefficient and low-level regeneration processes in tissue culture. The first transgenic stone fruit species have emerged from transformed hypocotyls. These great achievements were applauded by the scientific community contrary the fact that hypocotyl derived transgenic plants have no real breeding value. Tissue culture of different organs of valuable cultivars are recorded with an extremely low-level of regeneration in the literature. To improve the tissue culture basis of stone fruit plants an extensive tissue culture programme were launched and dozens of different media were compared including a series of hormone concentration in the tissue culture systems. Our continuous efforts were crowned by a very efficient method for achieving up to 30-40% regenerable petioles. Usually on a single petiole several well-separated meristems were induced. After 3-4 weeks of cultivation shoots were developed. The basic media K_2 were supplemented with 10g/L saccharose, 10g/L glucose and 10g/L maltose. The following plant hormones were used BAP 1mg/L, TDZ 1mg/L, 2-iP 1mg/L and IAA 0,1 mg/L concentrations. The Petri dishes were kept for 3 weeks in dark at a temperature 22℃ for 8 hours and 22-24℃ for 16 hours. The Petri dishes were sealed with Parafilm. The regeneration of the petioles were genotype independent and we were able to regenerate different plum cultivars with almost the same efficiency.

* Corresponding author, E-mail ; soos@abc.hu
Received Jun. 3, 1999 ; accepted Jun. 20, 1999

Introduction

Climate conditions in the Carpathian basin are extremely favourable for stone fruit cultivation. The wide varieties of peach, apricot, cherry, sour cherry, almond and plum are producing high quality fruits with remarkable market values based on their expressed traits. Cultivation of stone fruit species in this region has a long history, and can be demonstrated by plum as an example. The first certified plum species has been known since 1184, while our main and well-known Besztercei cultivars were mentioned already in 1523 (Surányi, 1998). Today almost all stone fruit cultivations are hampered by the plum pox virus epidemy in Europe. The negative impact of this virus disease on fruit production is so significant that special attention has been formulated by saying either someone could produce resistant varieties or have to forget those fruits in the near future. As conventional plant breeding was not able to make a major lead (i.e. producing virus resistant varieties) on the virus spread in Europe, an alternative solution had to be envisaged. The rapid development of molecular biology and its application in plant breeding as a precision breeding made it possible to elaborate strategies for producing virus resistant crops including stone fruit species (Wilson, 1993). Preliminary and significant results were obtained by French and Austrian research groups by using transformation of hypocotyls of plum and apricot species with coat protein gene of plum pox virus strains (Regner et al., 1992; Jacquet et al., 1998; Ravelonandro et al., 1992). Both groups have obtained several virus resistant lines, however their breeding values are in question, as the original

Table 1. Contents of media used for micropropagation and shoot proliferation of plum and apricot cultivars

	SZ	K1	K2(14)	K3(24)	BM(6)	QL(17)
NH_4NO_3	1650	400	830	800	500	400
$Ca(NO_3)$ $4H_2O$	-	600	295	556	250	200
$CaCl_2$ $2H_2O$	440	100	255	96	-	-
KNO_3	1900	900	1646	-	500	1800
$MgSO_4$ $7H_2O$	370	370	369	370	200	3600
KH_2PO_4	170	170	510	170	200	2700
K_2SO_4	-	-	-	990	-	-
H_3BO_3	3.0	3.1	6.2	6.2	1.0	1.0
$MnSO_4$ $4H_2O$	11.0	7.8	15.6	30.3	13.1	10.0
$ZnSO_4$ $7H_2O$	4.0	4.3	8.6	8.6	1.0	1.0
$NaMo_4$ $2H_2O$	0.3	0.125	0.25	0.25	-	-
$CuSO_4$ $5H_2O$	0.3	0.0125	0.025	0.25	0.3	0.3
$CoCl_2$ $6H_2O$	-	0.0125	0.025	-	-	-
KI	0.3	0.83	0.83	-	0.01	0.1
NaFeEDTA	36.7	45	55.4	36.7	25	36.7
myo-Inositol	500	500	150	100	100	50
Glutamin	-	100	-	-	-	-
Nicotinic acid	1.0	-	0.5	0.5	0.5	1.0
Pyridoxine	-	-	0.5	0.5	0.5	-
Thiamine	1.0	0.4	1.5	1.0	1.0	1.0
Ca-pantothenate	0.5	-	0.5	-	-	0.5
Biotin	0.1	-	0.01	-	-	0.1
Folic acid	0.01	-	-	-	-	0.01
Glycine	-	-	2.0	2.0	-	-
p-Aminobenzoic acid	1.0	-	-	-	-	1.0
Riboflavine	0.1	-	-	-	-	0.1
Ascorbic acid	-	0.1	-	-	-	-
Adenine hemisulphate	-	0.3	-	-	-	-

transformed tissue has no breeding values (Scorza et al., 1994; Laimer da Camara Machado et al., 1992; Mante et al., 1989; Mante et al., 1991). It is well known that transformation of woody plant tissues are very difficult and has not worked out yet which could be realised by that very few examples are known on successful transformation of those species.

In this report we are giving a detailed descriptions both on the tissue culture and regeneration of plum and apricot varieties and referring our genetic engineering results on inducing plum pox virus resistance on model poant as Nicotiana benthamiana (surányi, 1998).

Materials and Methods

Plant materials

Three cultivars of plum (Besztercei, Csacsanszka, Blue Free) and five cultivars of apricot (Ceglédi bí bor, Budapest, Harcot, Ceglédi kedves and 116/42 rootstock cultivar) were examined in this study. The basic, virus free shoot culture materials are maintained in the gene bank of Ornamental and Fruit Crop Research Institute, Budapest.

Micropropagation

To produce plant materials in proper condition for the regeneration experiments, six media with different macro- and microelement contents were compared (Table 1). Their pH were adjusted to 5.6 with KOH, prior to autoclaving and they were solidified with 0.65% Oxoid agar. The medium showing the best result was supplemented with different concentrations and combinations of sugars, auxins and cytokinins. All cultures were maintained in VEG Boxes (10 shoots/box) under a 25℃ light (16 h) / 20 ℃ dark (8 h) regime, being transferred to fresh media every 3 weeks.

Table 2. Different concentrations of carbonhydrates and benzyl aminopurine used.

Medium	Cytokinins (mg/L)						Auxins (mg/L)		$AgNO_3$ (mg/L)	R/E	R%
	2iP	BAP	BAPrib	TDZ	Zeatin	Kinetin	IAA	2,4-D			
KR28	1.0	1.0	-	1.0	-	-	0.1	-	-	108/410	26.3
KR28A1	1.0	1.0	-	1.0	-	-	0.1	-	1.0	37/100	37.0
KR28A2	1.0	1.0	-	1.0	-	-	0.1	-	2.5	49/100	49.0
KR28A3	1.0	1.0	-	1.0	-	-	0.1	-	5.0	13/100	13.0
KR28A4	1.0	1.0	-	1.0	-	-	0.1	-	10.0	10/100	10.0
KR28V2	2.0	2.0	-	2.0	-	-	0.2	-	-	18/ 80	22.5
KR28V4	4.0	4.0	-	4.0	-	-	0.4	-	-	19/ 80	23.8
KR32	1.0	1.0	-	0.5	-	-	0.1	-	-	0/ 50	0.0
KR33	1.0	1.0	1.0	-	-	-	0.1	-	-	0/ 50	0.0
KR34	2.0	1.0	-	1.0	-	-	0.1	-	-	58/150	38.7
KR35	2.0	1.0	-	0.5	-	-	0.1	-	-	10/ 60	16.7
KR36	2.0	2.0	-	1.0	-	-	0.1	-	-	6/ 60	10.0
KR37	2.0	1.0	-	1.0	-	-	-	0.25	-	0/ 60	0.0
KR38	1.0	1.0	-	-	-	1.0	0.1	-	-	0/ 50	0.0
KR39	1.0	1.0	-	-	1.0	-	0.1	-	-	0/ 50	0.0
KR40	1.0	-	-	1.0	-	1.0	0.1	-	-	0/ 50	0.0
KR41	1.0	1.0	-	1.0	-	1.0	0.1	-	-	0/ 50	0.0

Regeneration experiments

The first 3-4 expanded leaves with 2-3 mm long petioles were excised from the three-week-old proliferating shoots. A maximum of ten explants were placed in 100 mm Petri dishes containing the different regeneration media. The basic content of regeneration media was the same as of the medium chosen for micropropagation and they were supplemented with different concentrations and combinations of sugars and growth regulators detailed in Table 2. The plates were sealed with Parafilm. Cultures were initially incubated in the dark for 21 days at 25 ℃ (16 h) / 20 ℃ (8 h) and then maintained under light/dark cycle regime at the same temperature. Following regeneration, shoots were transferred to micropropagation media.

Results and Discussion

The main goal of this study was to develop an effective regeneration system based on shoot induction of vegetative tissue of plum and apricot. One of the pre-requisites for this system is producing suitable starting plant material as source of explants. The leaves expanded in the micropropagated shoots were the best candidate for this purpose. They can be produced in considerable amounts and homogene quantity.

Shoots of plum and apricot cultivars were micropropagated on six different media for 2 × 3 weeks and growing vigour and the condition of the cultures were compared. The best result was produced on the K_2 medium, which was selected for the further optimization. It was supplemented with variable concentration and combinations of sugars (sucrose: 30, 25, 20 g/L; sorbitol: 22 g/L and 15 g/L sucrose + 11 g/L sorbitol) (Marino et al., 1993) and growth regulators (Marino et al., 1993; Snir, 1984; Weiss et al., 1994). The variation containing 15 g/L sucrose, 11 g/L sorbitol, 0.1 mg/L BAP and 0.2 mg/L GA_3 proved to be the most suitable for micropropagation of all plum cultivars. The optimal medium for apricot culturing differed only in the BAP concentration, which was 0.2 mg/L. The shoots were devised and transferred to fresh media every three weeks and the fresh, fully expanded intact leaves arising from the upper 3-4 nodes were used as explants.

In preliminary regeneration experiments the B3 clone of Besztercei cultivar showed the best regenerating features, so this clone was included in the further optimization. The K_2 medium was used as basic medium. The sugar content optimization was carried on near the same growth regulation composition adding different combinations of maltose, glucose, sucrose and sorbitol. Most shoots were produced on the medium containing maltose, glucose and sucrose, 10 g/L of each, but the regeneration level did not exceed the 7-8 %. As a result of these experiments it has become clear, that shoots were produced only over certain hormones. Low level regeneration was occurred applying the combinations of BAP (2 mg/L) - IAA (0.1 mg/L) and TDZ (1 mg/L) - IAA (0.1 mg/L). There was no shoot induction in the presence of IBA, 2,4-D, BAPrib and zeatin. In

the next steps media containing the variations that of hormones resulted even in low level of shoots development were tested. An other cytokinin, the 2-iP was also applied. The medium KR28 (Table 2) designed in that way resulted in a great increasing in the regeneration efficiency, occasionally it reached the 27-32 %. The quantity of the regenerants were also changed: several, well separated shoots meristems were developed on a single petiolar (Plate 1). To reduce the effect of ethylene the KR28 medium was supplemented with $AgNO_3$. Using silver-nitrate in concentrations 1.0 and 2.5 mg/L the number of developing shoots was increased. However the developed shoots were mostly deformed and tend to be vitrificated. At higher concentrations the regeneration frequency was decreased. We have made attempts to change the growth regulator contents of KR28 medium with keeping or modifying the original hormone ratio (Table 2). Using the two- and fourfold hormone concentrations (KR28V2 and KR 28V4) the regeneration efficiency did not change essentially. TDZ proved to be necessary for effective shoot production. Reducing the TDZ concentration (KR32, KR35) or replacing it with BAPrib (KR33), kinetin (KR33), Kinetin (KR38) or zeatin (KR39), the regeneration rate was also decreased. The shoot production could be increased by higher level of 2-iP concentration (KR34 and KR35), while similar changes in BAP concentration(KR36) led to the opposite result.

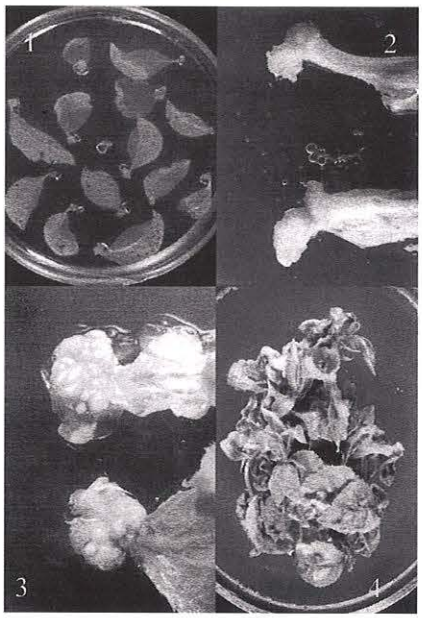

Plate 1. Regeneration steps of B3 clone of Besztercei plum; 1 and 2 callus formation at the end of petioles; 3 shoot initiation on calluses developed on the petioles; 4 regeneration of young shoots

This regeneration system has some essential factors. The first is the age and the quantity of the leaves used as explants. The frequency of shoot regeneration decreased with using older than 3 week-old leaves even if the symptoms of the senescence have not been seen. Similar observation was reported in connection with shoot regeneration from leaf discs or from cotyledons of peach. (Laimer et al., 1988; Mante et al., 1989). Our attempts to induce shoots from leaf discs were unsuccessful. Occasionally callus formation occurred in the cut edges of the leaves, but shoots developed only at the petiolar end. Explants with hurt blade usually became necrotic and the shoot induction was inhibited.

The other important factor was the presence of TDZ in the regeneration medium and the ratio of the suitable hormones. TDZ proved to be very effective for organogenesis of several *Prunus* species (Escalettes and Dosba, 1993; Laimer da Camara Machado and Laimer da Camara Machado, 1995b; Mante et al., 1989) and our experiments also verified the importance of this phenylurea derivative.

The regeneration ability of other plum and peach cultivars was also tested on the best regeneration media. The Blue free and Csacsanszka showed similar regeneration rate, however in the case of peach cultivars this system proved to be ineffective: the regeneration frequency did not exceed the 5%.

Based on the developed regeneration system we started *Agrobacterium*-mediated transformation experiments. Since the inoculation and the two days cocultivation with *Agrobacterium* strongly reduced the shoot induction caused early necrosis of the petiolar ends, considerable amount of explants are used for transformation. Although some shoots already were developed on the kanamycin containing selection media, further challenge to optimize the circumstances of the transformation method.

Acknowledgements

This work was supported by OMFB grant No: 94-97-44-0815. A. Wittner is grateful to UNESCO-BETCEN for awarding research fellowship. This work is a part of her Ph.D. work which was partly supported by SOROS Foundation-Hungary.

References

EscalettesV, Dosba F (1993) In vitro adventitious shoot regeneration from leaves of *Prunus* spp. Plant Science 90: 201-209

Jacquet C, Ravelonandro M, Bachelier JC, Dunez J (1998) High resistance to plum pox virus (PPV) in transgenic plants containing modified and truncated forms of PPV coat protein gene. Transgenic Research 7: 29-39

Jámbor-Benczúr E, Riffer AM (1990) In vitro propagation of *Phylodendron tuxtlanum* Bunting with benzylaminopurine.

Acta Agr Hung 39: 3-4

Laimer da Camara Machado M, Hanzer V, Mattanovich D, Himmler G, Katinger HWD (1988) regeneration of shoots from leaf discs and stem microcuttings of fruit trees as a tool for transformation. Acta Hortic 235: 85-92

Laimer da Camara Machado M, da Camara Machado A, Hanzer V, Weiss H, Regner F, Steinkellner H, Mattanovich D, Plail R, Knapp E, Kalthoff B, Kattinger H (1992) Regeneration of transgenic plants of Prunus armeniaca containing the coat protein gene of Plum Pox Virus. Plant Cell Reports 11: 25-29

Laimer da Camara Machado A, Laimer da Camara Machado M (1995b) Genetic transformation in *Prunus armeniaca* L. (apricot). In: Biotechnology in Agriculture and Forestry, Vol. 34, Plant Protoplasts and Genetic Engineering VI. (eds. Bajaj P.S.) 240-254

Mante S, Scorza R, Cordts JM (1989) Plant regeneration from cotyledons of Prunus persica, Prunus domestica and Prunus cerasus. Plant Cell Tiss Org Cult 19: 1-11

Mante S, Morgens PH, Scorza R, Cordts JM, Callahan AM (1991) *Agrobacterium*-mediated transformation of plum (Prunus domestica L.) hypocotyl slices and regeneration of transgenic plants. Bio/Technology 9: 853-857

Marino G, Bertazza G, Magnanini E, Altan AD (1993) Comparative effects of sorbitol and sucrose as main carbon energy sources in micropropagation of apricot. Plant Tiss Org Cult 235-244

Palkovics L, Wittner A, Balázs E (1995) Pathogen-derived resistance induced by integrated plum pox virus coat protein gene into plants of *Nicotiana benthamiana*. Acta Horticulturae 386: 311-317

Quoirin M, Lepoivre P, Boxus P (1977) Un premier bilan de 10 années de recherces sur les cultures de meristémes et la multiplication in vitro de fruitiers ligneux. C.R.Rech. Stn. Cult.Fruit.Maraichéres Gembloux 93-17

Ravelonandro M, Monsion M, Teycheney PY, Delbos R, Dunez J (1992) Construction of a chimeric viral gene expressing plum pox virus coat protein. Gene 120: 167-173

Regner F, da Camara Machado A, Laimer da Camara Machado M, Steinkellner H, Mattanovich D, Hanzer V, Weiss H, Katinger H (1992) Coat protein mediated resistance to Plum Pox Virus in *Nicotiana clevelandii* and *N. benthamiana*. Plant Cell Reports 11: 30-33

Scorza R, Ravelonandro M, Callahan AM, Cordts JM, Fuchs M, Dunez J, Gonsalves D (1994) Transgenic plums (*Prunus domestica* L.) express the plum pox virus coat protein gene Plant Cell Reports 14: 18-22

Snir I (1984) In vitro propagation of 'Canino' apricot. HortScience 19: 229-230

Surányi D (1998) Plum Cultivation in Hungary Hungarian Agricultural Research 4: 15-20

Weiss H, Hanzer V, Knapp E, da Camara Machado A, Katinger H, Laimer da Camara Machado M (1994) In vitro Vermehrung von *Prunus armeniaca*. Tagungsbericht Nutzbarmachung genetischer Ressourcen für Züchtung und Landschaftgestaltung, Pillnitz 1993 Vortr. P Flanzenz chtg 27: 322-326

Wilson TM (1993) Strategies to protect crop plants against viruses: Pathogen-derived resistance blossoms. Proc Natl Acad Sci USA 90: 3134-3141