Plant Cell Tiss Organ Cult (2008) 92:25–30
DOI 10.1007/s11240-007-9298-0

ORIGINAL PAPER

Rapid shoot regeneration from thin cell layer explants excised from petioles and hypocotyls in four cultivars of *Brassica napus* L.

Asma Ben Ghnaya · Gilbert Charles · Michel Branchard

Received: 20 April 2007 / Accepted: 9 September 2007 / Published online: 29 September 2007

Abstract The present work describes a procedure that allows for the easy and rapid induction of caulogenesis in four cultivars of *Brassica napus* L. from transversal Thin Cell Layers (tTCLs). In order to investigate the regeneration ability of this crop, the effects of genotype, explant source and culture medium were examined on shoot regeneration. The tTCL explants were excised from hypocotyl and petiole of 2-week-old seedlings and cultured on a solid basal MS medium supplemented with α-naphthaleneacetic acid (NAA: 0.1–0.4 mg l^{-1}), 6-benzylamino-purine (BAP: 1–4 mg l^{-1}) and sucrose (20–40 g l^{-1}). A significant genotypic effect was observed between the four cvs; Jumbo and Drakkar displayed higher capacities to produce shoots than Pactol and Cossair. Regeneration commenced earlier and the percentage of shoot-producing explants as well as the number of shoots per regenerating explant was greater. The comparison between the regeneration ability of different explants showed that the hypocotyls exhibited a high rate of shoot organogenesis when they were cultured on MS medium supplemented with 3 mg l^{-1} BAP, 0.3 mg l^{-1} NAA and 30 g l^{-1} sucrose. Adventitious shoot buds developed from 46% of the tTCLs, with a mean of 7.5 buds. Furthermore, the method was fast with shoot formation occurring by 7 days culture. Plantlets regenerated from all shoots and developed normally. The regenerated plants were fertile and identical to source plants.

Keywords Buds · Caulogenesis · Rapeseed · Regeneration · Thin cell layers (tTCLs)

Abbreviations

BAP	6-Benzylamino-purine
NAA	α-Naphthaleneacetic acid
MS	Murashige and Skoog's medium (1962)
PGRs	Plant growth regulators
tTCL(s)	Transverse thin cell layer(s)

Introduction

Brassica napus L. (oilseed rape, rapeseed) is one of the most important vegetable oil and protein-rich meal crops in the world. Its cultivation has increased tremendously during the last decade and, by now; it is the second largest contributor to the world supply of vegetable oil (Tang et al. 2003). In addition, the plant is able to produce biomass with added economical value. Hence, increasing its production is one of the

A. Ben Ghnaya · G. Charles (✉) · M. Branchard
Laboratoire de Biotechnologie et Physiologie Végétales, ISAMOR/ESMISAB-Université de Bretagne Occidentale, Technopôle Brest-Iroise, Plouzane 29280, France
e-mail: Gilbert.Charles@univ-brest.fr

A. Ben Ghnaya
e-mail: benghnaya_asma@yahoo.fr

most important challenges. To this effect, researchers focused on the genetic improvements.

In the past years, genetic improvements have mainly been achieved by conventional breeding methods, especially for F1 hybrid production. Genetic engineering also increased the possibilities for rape improvement (Charest et al. 1988), but a fast and reliable regeneration system is required for efficient *Agrobacterium*-mediated transfer of various agronomic and quality characters (Takasaki et al. 1997; Tang et al. 2003). Furthermore, uniform regeneration protocols would be desirable for maintenance of original genetic combinations or self-incompatible lines. In addition, efficient tissue culture systems provide the opportunity to understand mechanisms that control processes such as mutagenesis and transgenesis. These systems can also be used to increase genetic variability. For *B. napus*, it is known that shoot regeneration largely depends on genotype, explant source (Akasaka-Kennedy et al. 2005), PGR concentration and sucrose content (Nhut et al. 2003; Tang et al. 2003). Previous studies showed that organogenesis could be achieved from various explants: cotyledons (Narasimhulu and Chopra 1988), immature cotyledons (Turgut et al. 1998), hypocotyls (Khehra and Mathias 1992), stem sections (O'Neill et al. 1996) and longitudinal Thin Cell Layers (lTCLs) from stems (Klimaszewska and Keller 1985; Nhut et al. 2003). However, to the best of our knowledge, there is no report on plant regeneration system using tTCLs excised from hypocotyls or petioles of young plants cultivated in vitro.

The present work describes a shoot regeneration system using transversal Thin Cell Layers (tTCLs) isolated from four cultivars of *B. napus* L. The TCLs technology originated almost 30 years ago (Tran Thanh Van 1973). Since then, TCLs have been successfully used in the micropropagation of many plants including some recalcitrant ones such as *Lupinus* species (Mulin and Bellio-Spataru 2000) or *Spinacia oleracea* (Léguillon et al. 2003). The tTCls have also been successfully applied to *Lilium longiflorum* (Bui et al. 1999) or *Oryza sativa* L. (Nhut et al. 2000). In the present paper, factors influencing shoot regeneration from tTCLs are investigated in rapeseed. The effects of genotype, explant source, PGR concentration and sucrose content are studied to establish a highly efficient and fast regeneration system.

Materials and methods

Plant material

Four *B. napus* L. cultivars were used to evaluate shoot regeneration: Jumbo, Drakkar, Cossair and Pactol. These four different cvs are pure lines, genetically fixed, and were obtained by autofertilization.

Origin and preparation of tTCL explants

Seeds were surface-sterilized in 70% ethanol for 30 s, followed by immersion in calcium hypochlorite (5% w/v) added with two drops of Tween-20 for 30 min. The seeds were rinsed three times with sterile water upon sterilization and sown in test tubes on MS medium (containing 20 g l^{-1} of sucrose and solidified with agar (Kalys, HP 696) at 6.5 g l^{-1}). They were incubated under a photoperiod of 12 h (40 µmol m^{-2} s^{-1}) provided by cool white fluorescent lamps, with a 22/20°C thermoperiod (light/dark). Explants (tTCLs of 0.3–0.5 mm) were excised from hypocotyls and petioles of 2-week-old seedlings and placed in contact with the medium (25 ml) in 90 mm plastic Petri dishes (50 tTCls of one source each). For each treatment the experiment was repeated thrice ($N = 3$).

Culture medium

Hypocotyl and petiole tTCls were cultured on MS medium (comprising macronutrients, micronutrients and vitamins of Murashige and Skoog 1962) supplemented with different combinations of BAP (1–4 mg l^{-1}), NAA (0.1–0.4 mg l^{-1}) and sucrose (20–40 g l^{-1}). All media were solidified with agar (6.5 g l^{-1}), adjusted to pH 5.8 by 0.1 N NaOH and sterilized by autoclaving at 120°C and 1 kg cm^{-2} for 20 min.

Culture conditions

The cultures were incubated in the same conditions as previously described. The number of explants with shoot buds was scored after 2 weeks culture and the adventitious shoots formed per explant were counted. Well-developed shoots were separated and transferred

to MS medium lacking PGRs to induce rooting (under the same conditions used for germination). The rooted shoots were acclimatized under a 16 h photoperiod at 50 µmol. m^{-2} s^{-1} provided by cool white fluorescent lamps, with a 22/19°C thermoperiod (light/dark). The small plantlets were transferred in pots containing sterile compost, watered daily and fertilized with Hoagland's solution twice a week in a mist chamber for 10 days prior to transfer in a naturally-lighted greenhouse. The acclimatization experiments were repeated several times throughout the three last years to check the capacity for normal flowering and fertility.

Data analysis

The rate of caulogenesis and the number of shoots per organogenic explant was recorded from three replicates, each with 50 tTCLs per treatment. The values were compared by analysis of variance (ANOVA) and the differences among means (5% level of significance) were tested by the Duncan's Multiple Range Test using StatGraphics Plus 5.1.

Results

Typically, tTCL explants (Fig. 1b) from 2-week-old seedlings (Fig. 1a) swelled after 4–5 days of culture. Sometimes, a small amount of light-green callus proliferation was observed through the binocular glass on the subepidermal area. Shoot regeneration occurred from tTCL explants that appeared green and formed a peripheral crown of buds after 7–10 days (Fig. 2a). These shoots were generally associated with intense rhizogenesis. Non-regenerating explants could show either rhizogenesis only or browning and necrosis after 3 days of culture.

Results indicate that shoot regeneration ability is strongly influenced by the genotype. Considerable variation in shoot regeneration from tTCL explants was observed both between and within the *Brassica* cultivars. The first shoots were observed after 7 days on tTCLs of Jumbo and Drakkar in almost all PGR combinations screened (Fig. 2b) while they appeared after 3–4 weeks from tTCLs of Pactol and Cossair. In addition, the caulogenesis rate ranged from 6% for Cossair to 46.7 % for Jumbo with the highest number

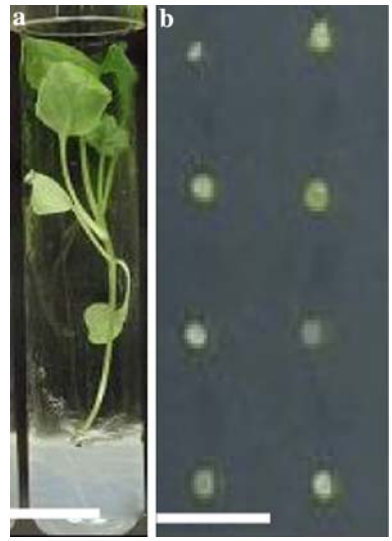

Fig. 1 Mother plant and tTCLs from rapeseed. (**a**) Fifteen-day-old seedlings of *B. napus* var. Jumbo (bar: 1.8 cm); (**b**) fresh hypocotyl tTCLs (bar: 1 cm)

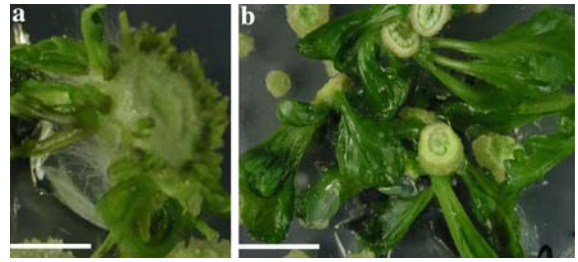

Fig. 2 Shoot regeneration from *B. napus* tTCLs after 2 weeks of culture on MS + 3 mg l^{-1} BAP + 0.3 mg l^{-1} NAA + 30 g l^{-1} sucrose. (**a**) Multiple shoots formed on hypocotyl tTCLs of Jumbo (crown of buds) (bar: 0.5 cm); (**b**) multiple shoots formed on hypocotyl tTCLs of Drakkar (bar: 1.2 cm)

of shoots per explant (7.5) (Table 1). Furthermore, the origin of tTCL explants also affected shoot regeneration: hypocotyl tTCLs best responded compared to petiole tTCLs for all the genotypes tested (Table 1). For Jumbo, the induction of adventitious buds from tTCLs of petiole reached 34.7% on MS medium added with 0.3 mg l^{-1} NAA, 3 mg l^{-1} BAP and 30 g l^{-1} sucrose.

In this study, four combinations of NAA and BAP were tested for each genotype and for each explant source. The best rate of plant regeneration was obtained with 0.3 mg l^{-1} NAA and 3 mg l^{-1} BAP for all genotypes and explants (Table 1). The least shoot regeneration frequency was observed for 0.1 mg l^{-1}

Table 1 Effect of genotype on in vitro organogenesis from tTCLs of petioles and hypocotyls

Genotype	Shoot regeneration frequency (%)		Number of shoots per tTCL	
	Petioles	Hypocotyls	Petioles	Hypocotyls
Jumbo	**34.7**^{a}	**46.7**^{a}	**5.5**^{a}	**7.5**^{a}
Drakkar	10.0^{c}	32.2^{b}	1.3^{bc}	2.5^{b}
Pactol	9.0^{c}	11.0^{c}	1.2^{bc}	2.0^{b}
Cossair	2.0^{d}	6.0^{cd}	1.0^{bc}	1.5^{c}

Data (percentage of tTCLs producing shoots and number of shoots per tTCL) were collected after 2 weeks of culture on medium supplemented with 0.3 mg l^{-1} NAA, 3 mg l^{-1} BAP and 30 g l^{-1} sucrose

The results were calculated from three replicated experiments, each with 50 tTCL explants per treatment. For each parameter, the values with different letters are significantly different (Duncan's test)

NAA and 1 mg l^{-1} BAP and for 0.4 mg l^{-1} NAA and 4 mg l^{-1} BAP (Table 2). With regard to sucrose concentration, 30 g l^{-1} was significantly beneficial to bud formation for the four varieties tested when cultured in the presence of 0.3 mg l^{-1} NAA and 3 mg l^{-1} BAP (Tables 1 and 2).

Finally, the shoots transferred in test tubes exhibited rooting and rapid development (Fig. 3a, b, c and d). When hardened just after rooting, regenerated plantlets transferred to pots in greenhouse showed a high rate of survival upon acclimatization (80–100%) (Fig. 4). The plants developed until flowering 8 weeks later (Fig. 5) and fertile seeds were harvested.

Discussion and conclusion

Based on the efficiency of the thin cell layer technology for the propagation of various plant species, this study was undertaken to achieve a high rate of regeneration in *B. napus* L. from tTCL explants. This technique promoted rapid and intensive shoot regeneration with shoot buds developing within 7 days for the most reactive genotype. These results compare favourably with recent studies of shoot regeneration of rapeseed from traditional explants (Tang et al. 2003; Akasaka-Kennedy et al. 2005) and from TCLs (Klimaszewska and Keller 1985; Nhut et al. 2003). Based on these two last

Table 2 Influence of PGRs and sucrose content on in vitro organogenesis from tTCLs of petioles and hypocotyls of *Brassica napus* cv. Jumbo

Sucrose (g l^{-1})	Growth regulators (mg l^{-1})		Shoot regeneration frequency (%)		Number of shoots per tTCLs	
	NAA	BAP	Petioles	Hypocotyls	Petioles	Hypocotyls
20	0.1	1	0.0^{e}	2.0^{d}	0.0^{c}	1.0^{c}
30	0.1	1	2.0^{d}	4.0^{d}	1.0^{c}	2.0^{b}
40	0.1	1	3.0^{d}	3.0^{d}	1.5^{c}	1.0^{c}
20	0.2	2	4.0^{d}	5.0^{d}	2.5^{b}	1.5^{c}
30	0.2	2	7.0^{cd}	7.0^{cd}	2.5^{b}	1.0^{c}
40	0.2	2	14.0^{c}	19.1^{c}	2.5^{b}	1.0^{c}
20	0.3	3	23.0^{b}	28.4^{b}	2.5^{b}	2.0^{b}
30	**0.3**	**3**	**34.7**^{a}	**46.7**^{a}	**5.5**^{a}	**7.5**^{a}
40	0.3	3	18.1^{c}	11.0^{c}	2.5^{b}	1.0^{c}
20	0.4	4	9.0^{cd}	3.0^{d}	2.0^{b}	1.0^{c}
30	0.4	4	3.0^{d}	2.0^{d}	1.5^{c}	1.0^{c}
40	0.4	4	0.0^{e}	2.0^{d}	0.0^{c}	1.5^{c}

Data (percentage of tTCLs producing shoots and number of shoots per tTCL) were collected after 2 weeks of culture

The results were calculated from three replicated experiments, each with 50 tTCL explants per treatment. For each parameter, the values with different letters are significantly different (Duncan's test)

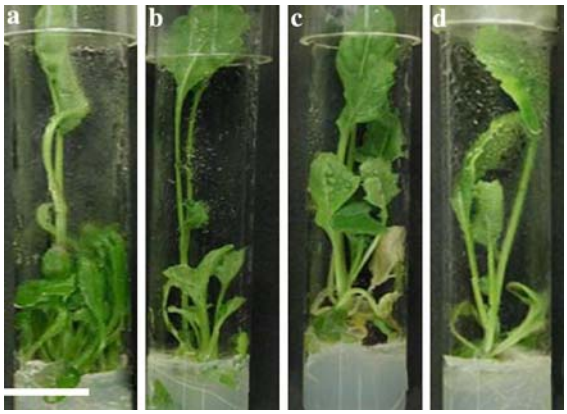

Fig. 3 Plants regenerated from hypocotyl tTCLs and transferred in test tubes on MS for development and rooting (bar: 1.8 cm): (**a**) Jumbo (**b**) Drakkar (**c**) Pactol (**d**) Cossair

Fig. 4 Rooted plants regenerated from hypocotyl tTCLs of Drakkar transplanted in pots for 1 week (bar: 4 cm)

references, using TCL explants provides a method for efficient bud regeneration but the explants were excised longitudinally and originated from 6-week-old flowering plants cultured in greenhouse. However, in our study, responsive TCL explants were excised transversally and from young axenic plants.

In our experiments, all the factors evaluated influenced shoot regeneration. In this respect, a strong genotypic effect was previously reported in *B. napus* tissue culture (Akasaka-Kennedy et al. 2005). Jumbo and Drakkar showed a greater capacity to produce shoots on the medium containing 0.3 mg l^{-1} NAA, 3 mg l^{-1} BAP and 30 g l^{-1} sucrose than Pactol and Cossair. The use of these four

Fig. 5 Plant regenerated from hypocotyl tTCL of Drakkar, flowering 8 weeks after transfer in pot (bar: 10 cm)

genotypes, two favourables and two recalcitrants within the same genus, may be a useful approach to conduct a genetic analysis of shoot regeneration (Julliard et al. 1992).

The source of tTCLs was also a critical factor in our experiments: for all genotypes, hypocotyl tTCLs best responded compared to petiole and exhibited the highest shoot regeneration frequency. Tang et al. (2003) showed that the PGR content and the sucrose concentration affected significantly the regeneration process from traditional explants as observed from tTCL explants in our study. Moreover, our results indicate a negative correlation especially for the most reactive genotype Jumbo, between the PGR content and the sucrose concentration: at low PGR content, shoot regeneration increased with the sucrose concentration and inversely (Table 2). Thus, sucrose may compensate for the lack of PGRs in culture medium for shoot regeneration.

Based on the factors evaluated, a fast regeneration protocol was achieved using in vitro cultivated tTCL explants which proved especially responsive for two of the four genotypes tested.

For further improvements, other factors could be taken into account, such as hormonal or light pretreatments (Julliard et al. 1992; Nhut et al. 2000), the age of the mother plant and the medium pH (Nhut et al. 2002), the addition of $AgNO_3$ (Akasaka-Kennedy et al. 2005) or of various sugars,

but also more specific factors such as tTCL explant thickness or position along the organ (Nhut et al. 2001).

In wider applications, tTCLs could be used as a tool for fundamental regeneration studies and for crop improvement through mutagenesis or transgenics. In addition to being an efficient regeneration process, Teixeira da Silva (2003) provided evidence of the capacity to efficiently produce non-chimeric transgenic plants using similar methods. Given this, tTCLs should provide a good system for the study of fundamental and applied aspects of regeneration and transformation of this main crop.

Acknowledgements The authors thank the National Institute of Agronomic Research (INRA) in Rennes (France) for furnishing the seeds of Jumbo and the Tunisian National Institute of Agronomic Research (INRAT) in Tunis (Tunisia) for those of Drakkar, Pactol and Cossair.

References

Akasaka-Kennedy Y, Yoshida H, Takahata Y (2005) Efficient plant regeneration from leaves of rapeseed (*Brassica napus* L.): the influence of $AgNO_3$ and genotype. Plant Cell Rep 24:649–654

Bui VL, Nhut DT, Tran Thanh Van K (1999) Plant production via shoot regeneration from thin cell layer pseudo-bulblets explants of *Lilium longiflorum in vitro*. C R Acad Sci 322:303–310

Charest PJ, Holbrook LA, Gabard J, Iyer VN, Miki BL (1988) *Agrobacterium*-mediated transformation of thin cell layer explants from *Brassica napus* L. Theor Appl Genet 75:438–445

Julliard J, Sossountzov L, Habricot Y, Pelletier G (1992) Hormonal requirement and tissue competency for shoot organogenesis in two cultivars of *Brassica napus*. Physiol Plant 84:521–530

Khehra GS, Mathias RJ (1992) The interaction of genotype, explant and media on the regeneration of shoots from complex explants of *Brassica napus*. J Exp Bot 43:1413–1418

Klimaszewska K, Keller WA (1985) High frequency plant regeneration from thin cell layer explants of *Brassica napus*. Plant Cell Tissue Organ Cult 4:183–197

Léguillon S, Charles G, Branchard M (2003) Plant regeneration from thin cell layers in *Spinacia oleracea*. Plant Cell Tissue Organ Cult 74:257–265

Mulin M, Bellio-Spataru A (2000) Organogenesis from hypocotyl thin cell layers of *Lupinus mutabilis* and *Lupinus albus*. Plant Growth Regul 30:177–183

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Narasimhulu SB, Chopra VL (1988) Species specific shoot regeneration response of cotyledonary explants of Brassicas. Plant Cell Rep 7:104–106

Nhut DT, Bui VL, Tran Thanh Van K (2000) Somatic embryogenesis and direct shoot regeneration of rice (*Oryza sativa* L.) using thin cell layer culture of apical meristematic tissue. J Plant Physiol 157:559–565

Nhut DT, Bui VL, Fukai S, Tanaka M, Tran Thanh Van K (2001) Effects of activated charcoal, explant size, explant position and sucrose concentration on plant and shoot regeneration of *Lilium longiflorum* via young stem culture. Plant Growth Regul 33:59–65

Nhut DT, Huong NDT, Bui VL, Teixeira da Silva JA, Fukai S, Tanaka M (2002) The change in shoot regeneration potential of protocorm-like bodies derived from *Lilium longiflorum* young stem explant exposed to medium volume, pH, light intensity and sucrose concentration pretreatment. J Hortic Sci Biotechnol 77:79–82

Nhut DT, Teixeira da Silva JA, Le BV, Tran Thanh Van K (2003) Thin cell layer studies of vegetable, leguminous and medicinal plants, Chapter 10. In: Nhut DT, Tran Thanh Van K, Le BV, Thorpe T (eds) Thin cell layer culture system: regeneration and transformation applications. Kluwer Academic Publishers, Dordrecht, pp 387–426

O'Neill CM, Arthur AE, Mathias RJ (1996) The effects of proline, thioproline and methyglyoxal-bis-(guanylhydrazone) on shoot regeneration frequencies from stem explants of *B. napus*. Plant Cell Rep 15:695–698

Takasaki T, Hatakeyama K, Ojima K, Watanabe M, Toriyama K, Hinata K (1997) Factors influencing *Agrobacterium*-mediated transformation of *Brassica rapa* L. Breed Sci 47:127–134

Tang GX, Zhou WJ, Li HZ, Mao BZ, He ZH, Yoneyama K (2003) Medium, explant and genotype factors influencing shoot regeneration in Oilseed *Brassica spp*. J Agron Crop Sci 189:351–358

Teixeira da Silva JA (2003) Thin cell layer technology in ornamental plant micropropagation and biotechnology. Afr J Biotechnol 2:683–691

Tran Thanh Van M (1973) In vitro control of de novo flower, bud, root and callus differentiation from excised epidermal tissues. Nature 246:44–45

Turgut K, Barghchi M, Scott R (1998) Efficient shoot regeneration and somatic embryogenesis from immature cotyledons of *Brassica napus* L. Plant Breed 117:503–504