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DIRECT SHOOT FORMATION *IN VITRO* CULTURE FROM COTYLEDON EXPLANTS OF *BRASSICA OLERACEA*

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Abstract

A study *in vitro* has been developed for efficient regeneration of shoots from *Brassica oleracea.* Using 4-day old cotyledons were used with petioles as explants and a combination of kinetin and Indol-Butyric Acid (IBA) in the regeneration media, after 2 weeks up to 80% of explants produced shoots in culture. a kinetin concentration of $2mgL^{-1}$ and IBA concentration of 1 mg $^{-1}$ were found in optimal conditions for regenerations. Regeneration potential had affected by light intensity. Rooting occurred simultaneously with shoot formation on these media and the resultant shoots could be rooted readily on minimal medium. This method would be widely applicable to *Brassica oleracea* cultivars where the genotype dependency was investigated and indicated. Histological studies after the initiation of meristematic activity in the cells indicated the development of multiple shoot from the petioles cut ends of the explants. Protocols involving cotyledonary petioles which described the compatible with previously reported *Agrobacterium* - mediated transformation.

Key Words *:* Cotyledon culture; tissue culture, shoot regeneration.

Introduction

High morphogenic potential from cells in the cut surface of an explant was developed by shoot regeneration system. *Agrobacterium* transformation were affected these cells, at the base of cotyledonary petioles and yielded large numbers of regenerating transgenic plants, the regeneration protocol was modified system initially established for *Brassica juncea* (Sharma *et al.,* 1990a ; Rihan, Al-Issawi *et al.*, 2012), *Brassica oleracea* (Al-Swedi 2013). The shoot regeneration potential of available cells within the explant have recently shown the major restriction in *Agrobacterium* - mediated transformation of *Brassica oleracea* (Al-Swedi, 2011; Moloney *et al.*, 1989). The same media and conditions were used in preliminary experiments as reported in Moloney *et al*., (1989) and Al Shamari, 2012) produced very few regenerates and therefore restricted the potential of this system for the production of transgenic plants from this species. We thus the cotyledonary petiole, investigated modifications for regeneration system and this system have improved for studies regeneration from a number of *Brassica oleracea* genotypes Histological carried out in this system to confirm the ontogeny and origin of multiple shoot formation

Principally tissue culture of rape cotyledon petioles were produced transgenic plants (Moloney *et al*., 1989; Damgaard *et al*., 1997; Zhang *et al*., 2006) and hypocotyl segments (Cardoza and Stewart 2003; Ramzan Khan *et al*., 2003; Peng *et al*., 2006; Zhang *et al*., 2006) by single *Agrobacterium*-mediated transformation. In general time-consuming and labour-intensive, comparatively expensive. A series of tissue culture using single *Agrobacterium*-mediated transformation containing co-cultivation shoot initiation, root inducing callus induction is essential to culture tissue cell and often 2-5 months are necessary to obtain complete transgenic plantlets in canola (Ponstein *et al*., 2002; Das *et al*., 2006). There are some adverse properties for tissue culture procedures such as somatic mutations (Al Shamari, Rihan *et al*., 2015) (Rakoczy-Trojanowska 2002) losing plants in transplanting, plant chimera, and complicated culture medium Therefore, it is favoured to build up oilseed rape bioreactor by developing a gene transfer of no-tissue culture.

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Materials and Methods

Plant materials

Seed sterilisation

The doubled haploid genotype DH AG1012 and cauliflower seeds. *Brassica oleracea* cultivars used in this study were supplied by John Innes centre in Norwich. Seeds were sterilized in 100% ethanol for 2 minutes followed by a solution of 10% commercial bleach with 1-2 drops of Tween for 10 minutes, followed by 3 rinses in sterile distilled water. The seeds were placed on germination medium comprising Murashige and Skoog (1962) salts and vitamins (MS), 3% sucrose and 0.8% phyto agar at a density of 20 seeds per plate and maintained at 23ºC in a 16h light/8h dark photoperiod.

Shoot regeneration

Cotyledons were excised from 4-day old seedlings (or from 4-6 day old seedlings in one experiment) so that they included ~2mm of petiole at the base. The petioles were embedded into the regeneration media tested here to a depth of ~2mm (Sharma, 1987). The sterile disposable petri dishes used were 90×15 mm for germination and regeneration and pots for rooting. Petri dishes were sealed with 1 layers of micropore tape and transferred to a 4°C cold room overnight before being transferred to a 23°C culture room under 16 hour day length.

Regeneration media comprised MS salts and vitamins, 2% sucrose, and various concentrations of kinetin and 3-indol butyric acid (IBA). Kinetin at $2mgL^{-1}$ was used in conjunction with $1mgL^{-1}$ IBA. Explants (10 per plate) were kept at 24ºC in a 16h light/8h dark photoperiod at a low light intensity. After 3 weeks, shoots and roots arising from the explants were counted. Percentage regeneration per plate (number of explants regenerating/total number of explants) was averaged for each treatment.

Transformation procedure

Single colonies of Agrobacterium tumefaciens strain EHA 105 containing a binary plasmid were grown overnight at 28°C in LB medium. For most of the work reported here, a binary plasmid pCGN 178 was used. This contains a CaMV 35S promoter and a neomycin phosphotransferase II (NPT II). Individual excised cotyledons were taken from the plates described above and the cut surface of their petioles was immersed into this bacterial suspension for a few seconds. They were immediately returned to the same MS plates from which they had been taken. The cotyledons were co-cultivated with the *Agrobacterium* for 72h. Chemical enhancers of virulence such as acetosyringone.

After co-cultivation, the sterile disposable petri dishes used were 90×20 mm. The cotyledons were transferred to regeneration medium comprising MS medium supplemented with 3% sucrose, 0.8% phyto agar, pH 5.8 and 500 mg/1 carbenicillin and 20 mg/1 kanamycin sulphate. Again the petioles were carefully embedded in the agar to a depth of 2 mm. Plating density was maintained at 10 explants per plate.

Selection and plant regeneration

The explants were maintained on regeneration medium, under light and temperature conditions specified above, for 2-3 weeks. During this time many shoots appeared on over half the explants with relatively little callus formation. Some of these shoots undergo bleaching by the fourth week of culture. The remaining green shoots were subcultured onto shoot elongation medium which was the same as regeneration medium. One or two weeks on this medium permitted the establishment of apical dominance from the shoot-clusters formed. The shoots so derived were transferred to 'rooting' medium containing MS medium, 3% sucrose, 2 mg/1 Indol butyric acid, 0.8% phyto agar and 500 mg/1 carbenicillin. No kanamycin was used at this stage as it was found that more rapid root establishment occurred without the selection agent while very few "escapes" actually succeeded in rooting after the two rounds of selection on regeneration and shoot elongation medium.

Results

Aspects affecting efficiency of regeneration

Media composition

Genotype 1012 and cauliflower (B. *oleracea)* cotyledon explants were used in primary experiments on regeneration. This was around 80% after 3 weeks using the protocol we formerly developed for *B. napus* regeneration (Moloney *et al., 1989).* When reducing the light intensity explant survival increased significantly and callus formation was preferred, but regeneration was still a rare event. Lowering the IBA concentration from 1 to 0.5mg/L increased regeneration to 5%. The most serious factor for development of shoot regeneration was the inclusion of kinetin in the medium. With kinetin, regeneration frequency increased to an optimum of ~70% at $2mgL^{-1}$ kinetin and $1mgL^{-1}$ IBA.

Fig. 1 illustrations the dose-response association of these explants to increasing kinetin concentrations. In the presence of kinetin some root formation was also stimulated, but this did not harmfully affect the appearance of shoot buds. The amount of IBA in the medium was moreover varied to establish the concentration of this growth regulator required for maximum root regeneration

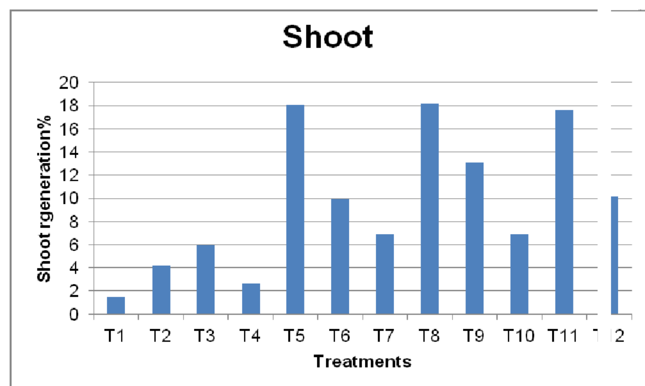

Fig. 1: Shoots regeneration from cotyledon explants of *B. oleracea* on kinetin and various concentrations of IBA. Data contains of 5 replicates, each with 10 cotyledons.

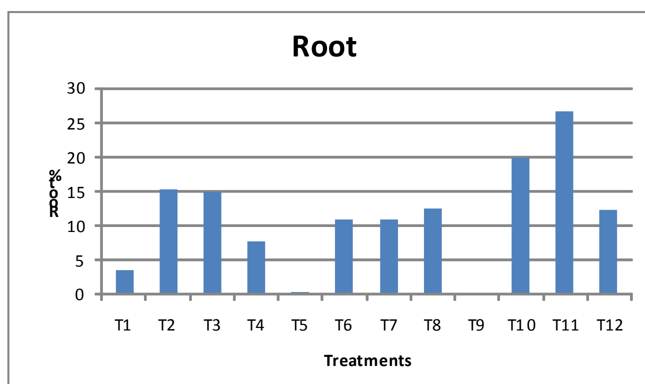

Fig. 2: Regeneration of roots from cotyledon explants of *B. Oleracea* on IBA and various concentrations of kinetin.

(Fig. 2). It was found that 2mgL^{-1} kinetin was active for shoot regeneration while IBA at a concentration of 1mgL^{-1} was best for root regeneration. The shoots arising on this medium were of a lesser quality often being more vitreous than those arising from explants on 1L^{-1} IBA. We decided to control whether the absolute concentrations of IBA and Kinetin were important or whether the auxin-cytokinin ratio was the major determinant for shoot regeneration. It was also difficult to obtain shoots with well-defined apical dominance at the higher IBA concentration. When we reduced the concentrations of kinetin and IBA while maintaining the same molar ratio we found a six-fold reduction in regeneration frequency after a five-fold reduction in absolute concentrations of these growth substances.

The existence of IBA in the medium caused the coexisting formation of shoots along with roots from the explant. Increasing in the production of shoots a relative to the production of roots caused by increase the concentration of kinetin from 1 to 2mgL^{-1} (Fig. 1).

Correspondingly, The shoot production relative to root production increase when the concentration of IBA increase from 0 to 1mgL^{-1} (Fig. 2). At the base of the regenerating shoots, the roots seemed and subsequently regenerated shoots transfer to a rooting medium was not required. As an alternative, the elongation of both roots and shoots permitted to arise by transfer it to a medium free of growth regulators. Actually, the explants conservation on regeneration medium for longer than two weeks was detrimental and caused in the vitrification of the newly formed shoots. The establishment of experiments were achieved whether there was a significant result on regeneration rate. Previously for member of the *Brassicaceae* such an effect has been reported (Chi and Pua, 1989; De Block *et al.,* 1989). Treatment with 500mgL^{-1} carbenicillin in this system, using optimal kinetin: IBA ratios,, as proposed by De Block *et al.,* (1989), in fact about 50% harmful regeneration frequencies was reduced. Furthermore at the cut end of the explant the resulted treatment was increased in callus formation and the production of clear shoots with an irregular morphology.

Explant age

Another essential factor in defining the rate of shoot regeneration is the age of explant. The obvious explants derived from seedlings older than 4 days by a stable decrease in shoot regeneration (Fig. 3). Whereas explants younger than 4 days old were established for their regeneration response, it has been shown for *B. oleracea* that a stable decline in regeneration efficiency happens from explants younger than 4 days old (Sharma *et al.,* 1990a). A decrease in shoot production by maintenance of 6-day-old explants under a high light intensity relative to root formation as compared to those incubated under the low light intensity system.

Table 1: Different combinations of growth regulator hormones.

T	(IBA)	(Kinetin)
1	0.0	0.0
2	1.0	0.0
3	0.1	0.0
4	0.2	0.0
5	0.0	1.0
6	0.05	1.0
7	0.1	1.0
8	1.0	3.0
9	0.0	3.0
10	0.05	3.0
11	1.0	2.0
12	0.2	2.0

Genotype effects

The regeneration of shoots from *B. oleracea* in several cultivars were tested for their organogenic response by determine whether the protocol presented here is genotype specific or of general utility. The 2 cultivars tested DH1012 indicated a 20% or greater shoot regeneration frequency than one recalcitrant cultivar cauliflower variety of *B. oleracea.* The frequency of regeneration in this line were improved in this experiments are currently started to improve by more modifications of the medium.

Growth of regenerated plants

As we are concerned the production of transgenic *B. oleracea* plants by using this regeneration system, normally for the capability of the regenerated shoots from set seed to mature is an important consideration. When regenerated shoots are placed on germination media, about 75% display apical dominance and these go on to flower and mature normally. After self-pollination, the mature plants can then be induced to set seed through the use of a sodium chloride solution to overcome the self-incompatibility existing in this species (Monteiro and Gabelman, 1988).

Callus induction

Brassica Oleracea species were isolated from hypocotyls, cotyledon and root to produced callus with high efficiency in media comprising high concentrations of the auxin 2, 4-D (0.5 mg/L). After 24 h, cell division began and after 48 h, 60% of the cells had divided. In these cases after 7 days, 92% of the cells had divided from hypocotyl protoplasts. A high frequency of regeneration obtaining by rapidly growing calli were transferred to media having a high cytokinin: auxin ratio as early as possible. The frequency of average regeneration for calli achieved from cotyledon protoplasts was 68%, while as many as 5% of the calli derived from root protoplasts.

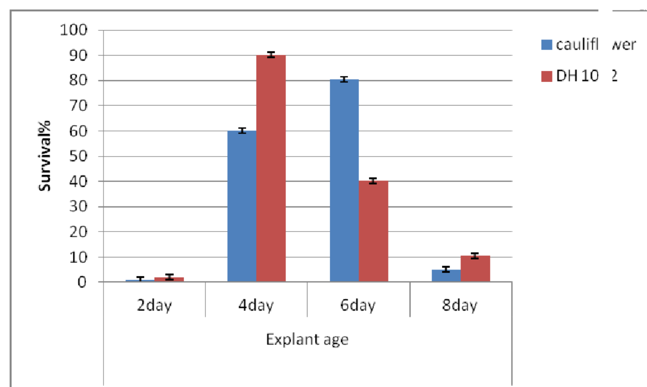

Fig. 3: Regeneration of shoots from cotyledon explants of DHG1012 & cauliflower *(Brassica oleracea*. Values are average obtained from donor seedlings of different ages.

Histogenesis of shoot primordial

In vitro The events of sequence that take place during shoot production from *B. oleracea* cotyledonary petioles is shown in fig. 5. The cortical cells of the cut end of the petioles were largely vacuolated at day 0, Whereas highly

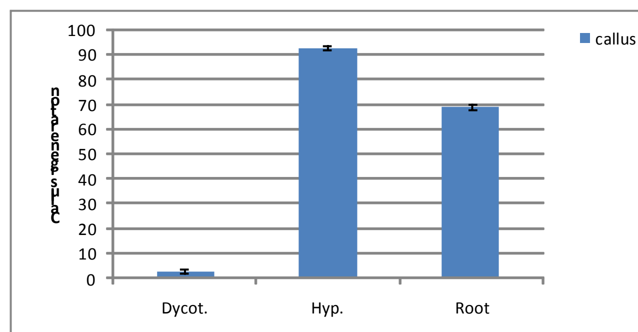

Fig. 4: Regeneration of callus from different parts of *Brassica oleracea (var. botrytis)* on 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1.0 mg/L indole3-butyric acid (IBA) and gel using 0.8% agar .Values are average Cotyledon ; hypocotyl and root. Data consists of 6 replicates each.

cytoplasmic cells with a prominent nucleus contributed by a vascular parenchyma. Starch grains were randomly dispersed in the cortical cells (Fig. 5A). random cell division activity had initiated in cells by day 2 on shoot regeneration medium, from the cut end of the petiole (Fig. 5B). The formation of multiple meristematic nodules by day 5 which resulted in, rapid cell divisions were limited to the peripheral areas (Fig. 5C). Shoot bud meristems gave rise to these nodules by day 7 (Fig. 5D), by day 11 which turn formed leaf primordia (Fig. 5E). By day 15 Shoot buds became macroscopic and by 21 clays fully differentiated shoots were expanded (Fig. 5F). As can be shown in fig. 5C, emerging numerous meristematic nodules is accomplished by each explant. This eventually leads to multiple shoot proliferation on a single explant.

Discussion

Constantly *B. oleracea* has confirmed to be one of the greatest recalcitrant of the *Brassica* species with concern to shoot regeneration *in-vitro* (Dietert *et al.,* 1982; Dunwell, 1981; Glimelius, 1984; Jain *et al.,* 1988; Murata and Orton, 1987; Narasimhulu and Chopra, 1988). Among numerous explants tested, shoot bud regeneration from cotyledons seems to have been the most active to date.

An optimal regeneration found *by* Narasimhulu and

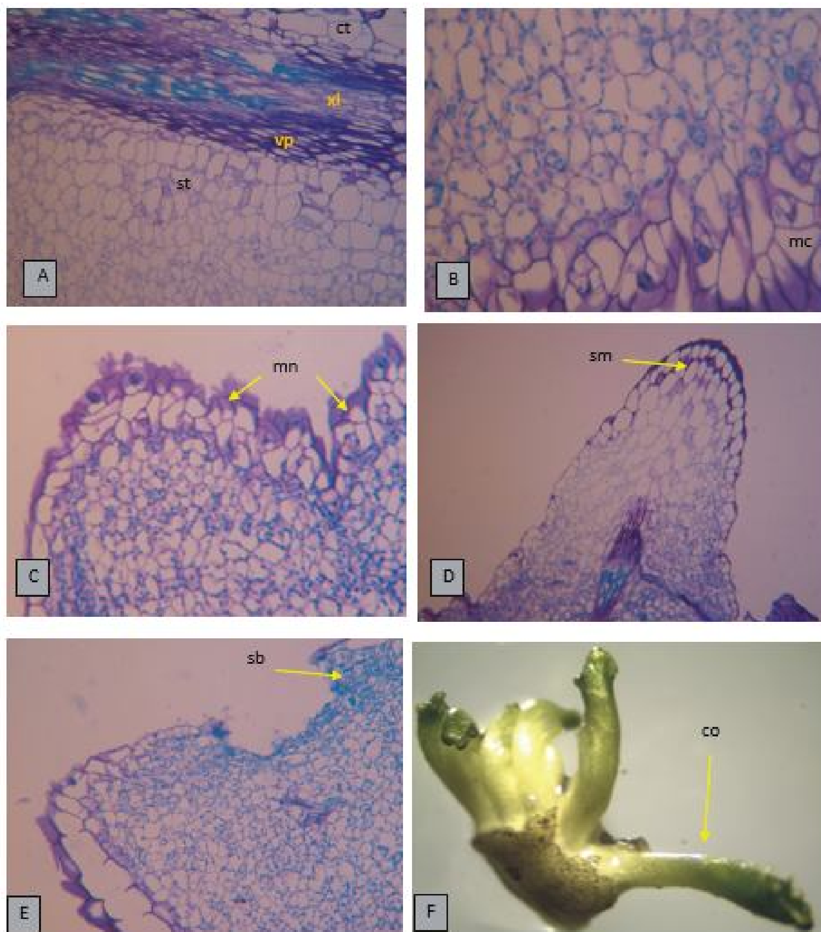

Fig. 5: Ontogeny (A-E) and morphology (F) of shoot bud histogenesis *of Brassica oleracea* from cotyledonary petioles. A. Longitudinal section cut of petiolar end at day 0 viewing the existence of highly cytoplasmic cells (arrow) with a prominent nucleus in the vascular bundles. Note the existence of randomly spread starch grains in the cortical cells (x378). B. At day 2, the petiole transverse section about 100gm from the cut end. The cells of the vascular parenchyma (arrow) are undertaking random cell divisions (x378). C. At day 5 transverse section of the petiolar cut end showing in the peripheral regions the formation of meristematic nodules (x189). D. At day 7 transverse section showing the improvement of an apical meristem of the petiolar cut end at the surface of the merlstematic nodule, designated by the existence of systematized dermal layers (x189). E. At day 11 transverse section viewing the improvement of a shoot meristem of the petiolar cut end along with leaf primordia (x189). F. After 21 days in culture formation of adventitious shoots from cotyledon explants. At day 14 The explant was transferred to hormone free mediurn from regeneration medium (x2). Abbreviation . [co-cotyledon; ct-cortex; mc meristematic cells; mn-meristematic nodule; sb-shoot bud; sm-shoot meristem; st-starch; vp-vascular parenchyma; xl-xylem].

Chopra (1988) that one cultivar of *B. oleracea* tested (32%) involving alike hormone and media combinations as were used here. Furthermore, Jain *et al.,* (1988) found the media containing auxin to be essential for regeneration of shoots from cotyledon explants of *B. oleracea* (up to 18%), on the other hand noted that IBA was unsuccessful cytokinin in this regard. In these two studies the differences in regeneration described could be due to variations in preparation of the explant, the genotype or culture conditions,. These presented and those results here are suggestive of the requirement for exogenous auxins in the regeneration medium for *B. oleracea*.

For shoot regeneration,this does not seem to be a requisite in some of the less recalcitrant members of the genus such as *B. junicea* (Sharma *et al.,* 1990a) *B. napus* (Moloney *et al.,* 1989), and *B. carinata* (Jaiswal *et al.,* 1987). It was surprising to find that the auxin concentrations increasing in the media affected a reduction in the frequency of root formation whereas increasing the frequency of shoot formation (Fig. 1). This is in contrast to what one might have probable based on the classical results of Skoog and Miller (1957) on organogenesis in tobacco. The earlier use of callus formation and shoot regeneration from cotyledonary explants of *B. oleracea* resulted in all parts of the explant including non-wounded surfaces.

In the regeneration material defined here occurs only at the base of the petiole at the cut end of the explant. The regeneration occurs very rapid with a minimum of callus formation is (2-3 weeks). It is clear the method that described here may be suitable for a wide variety of genotypes. Commonly, regeneration of *B. oleracea* has showed a high degree of genotype specificity (Jain *et al.,* 1988), which restrictions the number of varieties that can be regularly manipulated. Earlier work with *B. campestris* sp. *Chinensis* (Chi and Pua, 1989) and *B. olerace, B. napus* (De Block *et al.,* 1989) illustrated a significant improvement in regeneration rates in a variety of explant kinds. The result of shoots formation with an increase callus production and irregular morphology at the cut end of the explant.

The previous observed in *B. juncea* (Sharma and Bhojwani, 1990), the differentiation of multiple shoot in *B.*

oleracea was correspondingly followed by the development of multiple meristematic nodules. Within 2 days of culture these nodules, which formed, initiated in the cut end of the petiole from the meristematic activity of vascular parenchyma cells Such proximity to the cut surface would favour easy entrance to the meristematic cells by *Agrobacterium* during the initial, co-cultivation step of a transformation procedure. Consequently, the system designated here should advance itself readily to the transformation technique of Moloney *et al.,* (1989) which moreover as the explant uses cotyledonary petioles. We are presently trying to improve of numerous *B. oleracea* genotypes by this transformation technique.

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