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Effect of Pre-culture Irradiation and Explant Types on Efficiency of *Brassica napus* Genetic Transformation

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ABSRACT

The irradiated seeds of canola cv. Drakkar (*Brassica napus l.*) were germinated under aspect conditions, cotyledonary petioles and hypocotyls of 6 days old seedlings were used for Agrobacterium-mediated transformation. *Agrobacterium tumefaciens* has construct with the selectable marker gene (NPT II) and the desirable gene (HPPD). Direct and indirect shoot organogenesis were obtained from the both explants. Cotyledonary petioles was higher responded than hypocotyls with respective 26% and 14% of the explants producing NPT II-positive shoots after the selection on 50mg/l kanamycin. Calli might develop on and not in the agar medium were untransformation. This explains the higher number of escapes detected in hypocotyl explants than in experiments with cotyledons. The frequency of transformation plants as a function of indirect organogenesis was more than direct shoot regeneration from explants. The pre- irradiation with 75 Gy of gamma rays enhanced the genetic transformation frequencies by about 10 % as compared to that of the un-irradiated material. The obtained shoots were rooted and regenerated mature plants.

Key Words: Canola/ Transgenic/ Gamma rays/ Explants/ Regeneration.

Abbreviations*: BAP 6-Benzylaminopurine, MS Murashige and Skoog medium, NAA a-Naphthalene acetic acid, NPT II Neomycin phospho-transferaseII, HPPD Hydroxyphenylpyruvate dioxygenase, GM Genetically modified, PVP Polyvinylpyrrolidone, GA_3 Gebberellic acid, MES Moropholin ethansulfonic acid, IBA Indolbutric acid .*

INTRODUCTION

The introduction of genes into plants by means of genetic engineering is becoming an accepted technique in plant breeding. Genes derived from unrelated plant species and even other kingdoms (bacteria, fungi, animals), which would otherwise be inaccessible for the plant breeder, can with this new technology be introduced into their breeding programs. Although the techniques that pick up genes, to manipulate them and to introduce them into plants have evolved enormously in the last ten years, there are still a large number of unknowns. These are situated on each level of this very multi-disciplinary technology, starting from the function of the gene or promoter in plant physiological terms, through the understanding of the transgenic plant containing the manipulated gene in its genomic background. This has further interest in developing efficient plant transformation technologies to be able to concurly with the test and capture the value of these genes. If explants are used for direct and indirect transformations methods, a tissue culture period is necessary.

For rapeseed a number of different explants are used depending on the transformation protocol and the DNA delivery systems. Using *Agrobacterium* for the genetic transformation the following explants were used: stem intenode segments[1], thin cell layer[2] , hypo-cotyls[3,4,5,6], cotyledonary petioles[7], microspores or proembryos[8], inflorescence stalks[9]. For direct DNA transfer methods some of the most commonly used explants for the genetic transformation are mesophyll protoplast[10,11,12], hypocotyl protoplast[13], micro-spores[14,15] and stem sections[16]. However, transgenic canola plants have been produced at a relatively low frequency (1.5%)

through *Agrobacterium*-mediated gene transformation[17]. Therefore, this paper aims to increase efficient canola transformation technique using gamma radiation and different explants.

MATERIALS AND METHODS

Plant Material:

Seeds of canola cv. Drakkar (134 regenerations)were irradiated by gamma rays (^{60}Co) dose of 75 Gray at Nuclear Res.Center, Inshas. Seeds were obtained from Dr. W. Friedt, Institute of Crop Science and Plant Breeding, Justus-Liebig-University, Giessen, Germany and the work has been done in his lab. The irradiated seeds as well as un-irradiated were surface sterilized for 5 min in 70% ethanol, then for further 10 min in 3% NaOCL solution, followed by three rinses in sterile distilled water. These seeds were germinated under aseptic conditions on A_1 medium in jars for 6 days[4] in dark. The germination medium contained 1/2-strength MS macro- and microelements[18], 15 g/1 sucrose, and 8 g/l agar buffered to pH 5.8

Agrobacterium Preparation:

Agrobacterium tumefaciens strain harbouring the binary vector with the gene of interest (HPPD) and the neomycin phosphotransferase (NPT II) as a selectable marker was spread onto solidified YEB medium (1g/l Yeast extract, 5 g/l Beef extract and 5 g/l peptone) supplemented with 50 mg/l kanamycin, 30 mg/l chloromophyncol, 100 mg/l rifmpicin and 2 mM magnesium sulfate, over 3 days in dark in order to obtain single colonies. A volume of 3 ml of liquid YEB medium was inoculated with one colony and grew overnight on a rotary shaker at 28° C and 200 rpm. This overnight culture is directly used for the co-cultivation step.

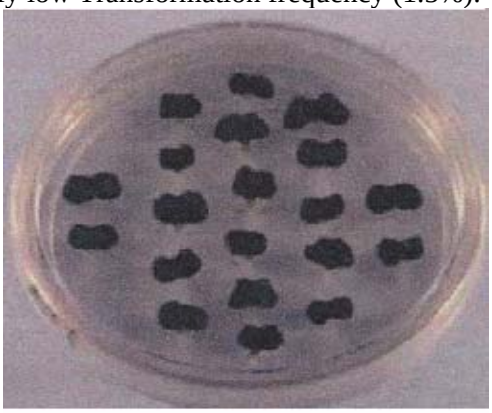
Co-cultivation, Selection and Regeneration:

Cotyledonary petioles and hypocotyl segments (1 cm long) from 6-day-old seedlings were used as explants in the transformation experiments. Hypocotyl explants were treated with 20 ul *Agrobacterium tumefaciens* of the over night culture for 3 days in 10 ml liquid A_2 medium (co-cultivation) using 9 cm Petri dishes. A_2 medium supplemented with 1 mg/l BAP, 0.1 mg/l NAA, 0.01 mg/l GA_3 and 0.5 g/l MES. While, cotyledonary petioles were dipped into the dilute *Agrobacterium* suspension for 1 second and place the cotyledons on co-cultivation medium, pushing the cotyledons into the soft agar medium. 20- Cotyledons were placed on each plate. The plates were wrapped by parafilm and incubated in the growth room (24^oC) for 3 days. Monitor the plates daily and if cloudiness around the tissue appeared indicating *Agrobacterium* growth, it should be transferred immediately to selection medium in growth room with 16 h/ 8 h photoperiod, 24°C with a light intensity of 500 $UEm^{-2}s^{-1}$. Following co-cultivation, the hypocotyl explants were placed on Petri dishes (2 cm high and 14.5 cm in diameter; 20-25 explants per dish) containing A_3 selection medium[4] with 50 mg/1 Kanamycin (as a selective agent),125 mg/l Betabactyl, 0.5g/l PVP, 40mg/l Adeninsulfat and 5mg/l Silver nitrate. From 80 to 100 explants with three replicates were used for each treatment (two explant types and two irradiation doses (0 and 75 Gy). Further sub-cultivation of the explants was conducted on the selection medium (A_3) at intervals of two weeks. After 6-8 weeks of selection the formed shoots were transferred to A_4 shoot elongation medium with 15 mg/1 Kanamycin and 62.5 mg/l Betabactyl. After 4 weeks, elongated shoots (longer than 1 cm) were then transferred to A_5 rooting medium contained 1/2 MS, 0.1mg/l IBA, 25mg/l Betabactyl and 15 mg/1 Kanamycin. Three to five were cultivated per jar (100 ml) and the rooting plantlets were transferred to the soil in green house to arise T_1 seeds. Transformation frequencies were expressed as the percentage of Gus positive shoots/total number of explants evaluated.

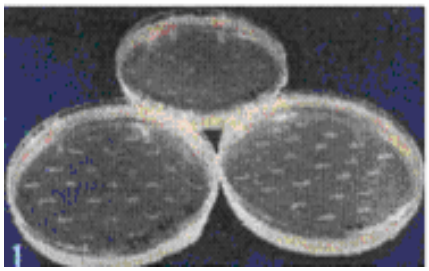
RESULTS AND DISCUSSION

1- Explant Types:

With cotyledonary petiole explants, calli developed on and not in the agar medium, as the case with hypocotyl explants were untransformation. This might explain the higher number of escapes detected in hypocotyl explants than in experiments with cotyledonary petioles (Fig.1). In this respect[17] has shown that B. *carinata* tissues of plantlets in vitro grown are also highly regenerative with relatively low Transformation frequency (1.5%).



A

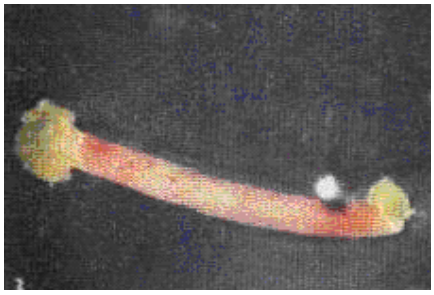


B C

Fig.(1): Selective medium supplemented with 50 mg/l Kanamycin as selective agents (A, Cotyledons and B , Hypocotyls) C, Direct Shoot organogenesis from hypocotyls

The direct developments of shoots from both cotyledon and hypo-cotyl explants within the first week (direct organogenesis) were more than 50% of the explants yielding shoots. However, the shoots indirectly proliferated from callus at the cut edges of the explants (indirect organogenesis) were about 30% of the explants yielding shoots. While only one genotype was used in this study, a preliminary survey of seven B. *carinata* genotypes revealed that all were highly responsive [19]. Other studies have previously confirmed the responsiveness of B. *carinata* seedling explants to tissue culture[20,21]. In the three species (B. *napus*, B. *rapa*, and B. *juncea*), adventitious shoots are produced directly from the explants without intervening callus formation and appear to be derived from the meristematic activity of vascular parenchyma cells present in the explants[7,22,23]. In the present study, however, most of the B. *napus*, shoot buds developing directly from the explants within the first week of culture were not transformed (Fig.1C). Most of the transformed B. *napus* shoots were, in fact, derived from the proliferating callus (Fig.2). It seems that dedifferentiation of explant cells was necessary for efficient transformation of B. *napus.* Such dedifferentiated cells appear to be highly competent for transformation such as in *tobacco*[24] and *arabidopsis*[25]. Some investigators[22,26] stated that to obtain transformation of shoots regenerating directly from the explants, it is essential that the totipotent cells be competent

for transformation and that they are accessible to *Agrobacterium* during co- cultivation. One week after co-cultivation, transformed cells were observed as green spots in callus on the cut edge of explants in the presence of 50 mg/l kanamycin (selective agent) as shown in Fig (2 A). After 2 weeks these spots developed into green calli. Most green calli developed in agar. However, other calli developed above the medium and produced green shoots were mostly NPT-II negative. Untrans-formed white calli (0.5-1cm^2) and white (bleached) shoots also developed on the selection medium containing 50 mg/1 kanamycin (Fig. 2C). Over 1 month, green calli developed into numerous green shoots with approximately 26% of the explants producing NPT II-positive shoots were detected (Table1).



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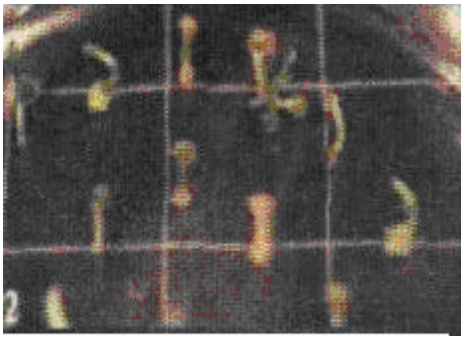

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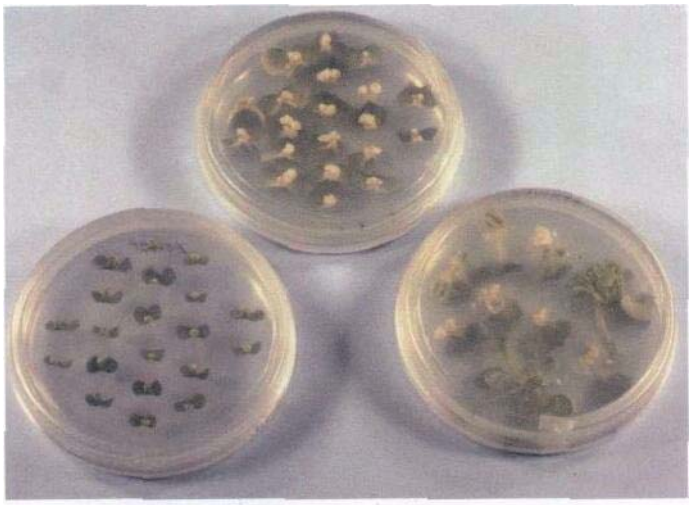

C

Fig (2) : (A) Green spots in callus on the cut edge of explants after a week in the presence of 50 mg/l kanamycin (selective agent).
After one month in selection medium with 50 mg/l kanamycin, proliferation of transformed shoots from hypocotyls (B) and cotyledons (C)

Table (1): **Effect of seeds pre-culture irradiation derived explants from irradiated seeds with 75 Gy of gamma rays on transformation frequencies in cotyledonary petiole explants of *Brassica napus*.**

Number of explants evaluated	Preculture irradiation	NPT II positive shoots %
240	-	26
235	+	38

In hypocotyl explants, green calli developed at the cut ends after 2 weeks on selection medium, and shoots emerged after 3-4 weeks, with approximately 14% of the explants producing NPT II positive shoots were obtained (Table 2).

Table (2): **Effect of seeds preculture irradiation derived explants from irradiated seeds with 75 Gy of gamma rays) on transformation frequencies in hypocotyl explants of *Brassica napus.***

Number of explants evaluated	Pre-culture irradiation	NPT II positive shoots %
285	-	14
294	+	22

2- Effects of pre- culture irradiation on the frequency of transformation plants:

The treatment of B. *napus* seeds with 75 Gy of gamma rays before culturing increased the transformation efficiency by about 12 % for cotyledonary petioles and 8 % for hypocotyls (Table1 and 2). One of the important factors that contributed to the high transformation frequency of B. *napus* was the ability to control the hypersensitive response of explants induced by cocultivation with *Agrobacterium*. The radiation may be developed compounds, which induce the expression of the *vir* genes of the *Agrobacterium* .

Wounding of tissue culture explants before inoculating with *Agro- bacterium* has been considered to be necessary for transformation as it is required for the synthesis of compounds which induce the expression of the *vir* genes of the *Agrobacterium*[27]. Although wounding is necessary for release of phenolic compounds and activating *Agro-bacterium*, released phenolics could also induce tissue browning and death, thereby inhibiting regeneration from transformed tissue. This hypersensitive reaction has been regarded as a defense reaction against *Agrobacterium* [28]. To reduce tissue damage by bacteria, a feeder layer of tobacco cells was used to obtain transformation of *Arabidopsis* leaf discs [29]. Another critical factor in achieving high frequencies of transformation in B. *carinata* is related to preculture of explants for 2 days on regeneration medium prior to co-cultivation [30]. Pre-culture of explants was previously reported with hypocotyl explants of B. *napus* and B. *rapa* [31, 32], but apparently with no influence on transformation frequency.

3- Regeneration:

After 6-8 weeks in selection media, the green shoots were translocation to elongation medium, low concentrations of BAP (0.0025mg/l), Betabactyle (62.5 mg/l) , 15 mg/l kanamycin and without auxin to obtain normal shoots (Fig. 3A). After about month, elongated shoots were placed on rooting half MS medium with 0.1 mg/l IBA, 25 mg/l Betabactyl and 15 mg/l Kanamycin for further selection

(Fg.3B).Rooted shoots were transferred to soil in the greenhouse after about 4 weeks to arise R_o-plants (Fig 4) which give R_1 –seeds.

A B

Fig (3): **Regeneration medium with 15 mg/l kanamycin**
(A) Shoot elongation after 4 weeks. (B) Rooting plantlets

A B

Fig (4): **NPT II positive plants**
(A) Genetically modified plant (GM) in soil
(B) Mature transgenic plants in greenhouse

CONCLUSION

1. The cotyledonary explant produced more transgenic plants than hypocotyls explant.
2. The calli grown on not in agar of selective medium were untransformation.
3. The canola genetic transformation frequency was enhanced by the pre-culture irradiation of seeds with 75 Gy for the both explants.

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