Optimization of In vitro Regeneration Protocol for Some Spring and Winter Cultivars of Canola Using Different Explants

Faeze Rezaei, Ebrahim Dorani Uliaie* and Mostafa Valizadeh
Dept. of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
dorani@tabrizu.ac.ir*; +98 4113392031

Abstract

Canola (Brassica napus) is the second largest oilseed crop in the world and it is one of the first crops to be genetically engineered. Beside, the classic breeding methods, the exploitation of plant genetic engineering has increased scientist’s hope to improve performance of canola under biotic and abiotic conditions. Factors influencing reliable shoot regeneration from different explants of rapeseed were examined in this study. The direct shoot regeneration ability of four winter (Licord, Opera, SLM046 and Karaj3) and three spring cultivars (RGS003, Sarigol and PF-7045) of canola in basal MS-medium supplemented by one hormonal level of BAP in combination with three levels of NAA and presence or absence of AgNO₃ were evaluated using cotyledon and hypocotyl explants in a factorial experiment. The difference between genotypes and NAA concentration were significant for regenerated explants and number of shoot per explants in both explants, except for number of shoot from hypocotyls. The highest percent of regeneration and the number of shoots per explant were obtained in SLM046 and PF-7045 cultivars with 0.1 mg/L NAA for cotyledons and RGS003 and Opera cultivars with 1 mg/L NAA for hypocotyls. Addition of AgNO₃ to medium was significantly effective for shoot regeneration on all genotypes. The effect of genotype and plant growth regulators was significant.

Keywords: Canola, shoot regeneration, cultivars, cotyledon, hypocotyls, genotype, plant growth regulators.

Introduction

Canola (Brassica napus L.) is one of the world’s most important sources of vegetable oil (Ramzan Khan et al., 2002) and it is the second largest oilseed crop after soybean in global oil production (FAO, 2011). It is a member of the family Brassicaceae. It is a winter or spring crop and is amenable to growth in cold climates. Canola oil is widely used as cooking oil, salad oil and making margarine. Off all edible vegetable widely available today, it has the lowest saturated fat content, making it appealing to health-conscious consumer. Canola meal, which is the leftover product of the seeds after extracting oil, is used as a protein supplement in dairy, beef, swine and poultry rations and is recognized for its consistent high quality and value. With the increasing demand for canola oil and the need to meet the demands of consumers, there is more research being pursued in improving canola breeding. Conventional breeding techniques are time consuming and laborious. An alternative way to trait improvement in canola is genetic engineering which reduces the time needed to develop a new variety. Genetic engineering approaches in canola have mainly focused on improving oil quality or making it herbicide tolerance and these plants are now commercially available (Lu et al., 2011). Transgenic canola is estimated to occupy about 8.2 M ha in the world (James, 2012). Biotechnological improvement, however, require extensive tissue culture work and efficient regeneration protocols. Many reports have been published in tissue culture and genetic engineering of canola. Although B. napus is considered to be suitable for various kinds of tissue culture and transformation techniques, but almost all reports till now are about a limited number of genotypes (Maheshwari et al., 2011). Cell and tissue culture that are related to variability and selection efficiency are two essential components of molecular breeding. Spring type canola varieties have proven to be most amenable for regeneration and transformation, but the European winter cultivars giving high yield because of tolerance to sub-optimal conditions and disease (Light et al., 2005).

Regeneration in canolais highly variable and there are several reports on shoot regeneration from explants derived from seedlings or mature plant of B. napus (Dunwell, 1981). Various explants have been used for regeneration experiments in canola are stem sections (Pua et al., 1991), stem thin cell layer (Klimaszewska and Keller, 1985), leaf discs (Dunwell, 1981), roots (Sharma and Thorpe, 1989), cotyledons (Moloney et al., 1989) and hypocotyls (Phogat et al., 2000). Among various systems used for canola transformation, Agrobacterium-mediated transformation used hypocotyl and cotyledon has been commonly employed because of the high regeneration capacity of these explants (khan et al., 2003).
In vitro plant regeneration has been found to depend on many factors, including: genotype explants type, composition of basic medium, growth regulators, gelling agent, light intensity and quality, photoperiod, temperature, cultivation vessels and vessel covers (Reed, 1999). Keeping these factors in view, the main aim of the present study was to encompass the effects of growth regulator, explant and genotype on in vitro regeneration in canola cultivars grown in Iran which can be exploited for genetic improvement programs.

Materials and methods
Collection of seeds: Seeds of three different spring cultivars including Rgs003, Sarigol and PF-7045 and four winter cultivars including Licord, Opera, SLM046 and Karaj3 were obtained from Agricultural Research Organization branches of Tabriz, Iran.

In vitro regeneration: Seeds were surface sterilized by 5% sodium hypochlorite solution for 10 min on the shaker and washed three times with sterile water. Thirty seeds were placed on petri dishes (90 mm dia) containing half-strength MS-medium (Murashige and skoog, 1962), cultured and germinated in growth room. Culture conditions were 23-25°C, 16 h photoperiod at 48 mol m⁻² s⁻¹. Cotyledons and hypocotyls were excised from 6 and 10 d old seedlings and were cultured in Murashige and skoog medium supplemented with BAP (1-benzylaminopurine) (3 mg/L), NAA (1-naphtalene acetic acid) (0, 0.1 and 1 mg/L) and AgNO₃ (0 and 3 mg/L). Experiments were carried out as factorial experiment based on completely randomized design in three replications and 20 explants in each replicate (petri dishes). Cultures were kept in a controlled environment growth room under same conditions. After one month, explants were subcultured to new same media and after two months, the regeneration frequency of explants and also number of regenerated shoots were counted and the results were analyzed by SPSS and MSTATC software after data transformation for obtaining normal distribution.

Results and discussion
Media composition: Regeneration frequency and number of regenerated shoots were different depending upon the hormonal combination, type of explants and cultivars. There was direct shoot regeneration in some treatments and indirect shoot regeneration from callus in others.

Regeneration from cotyledons: The frequency of regeneration capacity from cotyledons was different between genotypes, the highest regeneration was observed in PF-7045 (56.94%) followed by Licord and SLM046 and the lowest regeneration was observed in Karaj3 (20.23%). Presence of NAA in combination with BAP improved regeneration efficiency in all cultivars. Between different levels of NAA, 0.1 mg/L was the most effective concentration.

Interaction between cultivar and NAA concentration for shoot regeneration was significant and showed that PF-7045 in presence of 0.1 mg/L NAA had the highest frequency of regeneration from cotyledonary explants. In Licord and Karaj3, increasing of NAA concentration in culture medium, increased shoot regeneration and also in some cultivars such as RGS003 and in Sarigol absence of NAA in medium decreased regeneration efficiency even near zero. Generally, 0.1 mg/L NAA caused highest rate of regeneration in the most of cultivars compared with other levels of this growth regulator (Fig. 1).

Values with same letter are not different at p<0.05.

**Fig. 1.** Response of cotyledon explants of different cultivars for regeneration in MS-media supplemented with different levels of NAA.

**Fig. 2.** Average number of shoots regenerated from each cotyledon in different cultivars.

**Fig. 3.** Average number of shoots regenerated from each cotyledon with different levels of NAA.
The number of shoot regenerated from cotyledons showed that cultivars, SLM046, PF-7045 and Licord produced highest number of shoots respectively. RGS003 had the lowest number of shoots. Furthermore, the highest number of shoots was observed in media with 0.1 mg/L NAA (Fig. 2 and 3).

Regeneration from hypocotyls: RGS003 cultivar revealed the best response in the number of regenerated explants from hypocotyls, followed by Opera and Sarigol. The lowest regeneration frequency was observed in PF-7045. NAA effect on regeneration showed that 1 and 0 mg/L of this hormone, gave the highest and lowest regeneration respectively and there wasn’t significant difference between two levels of NAA, 1 and 0.1 mg/L, but presence and absence of NAA in regeneration media were significantly different. Interaction of cultivar × NAA was significant in 0.01 or 0.05 mg/L. The highest frequency of regeneration was noted in RGS003 in the medium supplemented with 0.1 mg/L NAA and the lowest (0%) was observed in RGS003 with medium without NAA and also in PF-7045 with medium supplemented with 0.1 mg/L NAA. In most of cultivars, the lowest rate of regeneration occurred in media without NAA, but there wasn’t significant different between two levels (1 and 0.1 mg/L) of NAA. So that, in Opera, regeneration percentage was completely same in both concentrations (0 and 0.1 mg/L) of NAA (Fig. 4). For number of shoots per hypocotyl explants, it was observed that in none of cultivars, different levels of NAA and cultivar × NAA interaction effects were not significant. In all experiments in this study, BAP was used in a fixed level of 3 mg/L in all media and had a good effect on regeneration in most of cultivars. Important role of BAP for development of shoots is already reported (Moloney et al., 1989). Ono et al. (1994) showed that the critical factor for shoot regeneration was the presence of BAP in the medium. The highest frequency of shoot regeneration (70%) was obtained in the presence of 4 mg/L BAP. Excluding BAP from the medium caused no induction of shoot regeneration. Guo et al. (2005) reported that the requirement of NAA for regeneration from cotyledons of B. juncea and observed that using NAA in low concentrations increased shoot regeneration significantly in all of used cultivars that are same with results of this study, so that the best concentration of NAA in the most of cultures was 0.1 mg/L. In the study by Burbulis et al. (2009), the use of NAA in combination with BAP resulted in significant increase in shoot formation frequency from hypocotyls of canola in most of used cultivars.

Effect of genotype on shoot regeneration: The frequency of regeneration capacity from cotyledons was different between genotypes, the highest regeneration was observed in PF-7045 (56.94%) followed by Licord and SLM046 and the lowest regeneration was observed in Karaj3 (20.23%).

The regeneration in B. napus is strongly variable and genotype depended. Ono et al. (1994) tested 100 cultivars and observed a huge variation from 0% to 91%. So genotype is a limiting factor in Brassica tissue culture and regeneration that limits manipulating and improving the germplasm (Cardoza and Stewart, 2004). Not only regeneration potential is different in genotypes, but various explants in a genotype aren’t same (Szule and Drozdowska, 1997), which is consistent with the findings of our experiment.

Burbulis et al. (2009) tested 10 commercial cultivars of canola in order to find the regeneration capacity and revealed that shoot regeneration rate is different in various genotypes and medium compounds. Also Zeynali et al. (2010) reported that organogenesis in B. napus occurred in medium supplemented with different concentrations of growth regulators, which is significantly affected by genotype and hormone levels. Uliaei et al. (2008) observed remarkable variation in regeneration ability from cotyledon explants of used genotypes of canola. The same results were achieved for Brassica campestris and B. napus by using leaf explants (Akasaka-Kennedy et al., 2005).

Effect of AgNO3 on shoot regeneration: AgNO3 was used in two concentrations (0 and 3 mg/L) in order to study its effect on regeneration from explants and its presence showed clearly a positive effect on shoot regeneration (Fig. 5). Also the presence of NAA had a significant effect on regeneration compared with its absence. The results of this study showed the positive effect of NAA and AgNO3 on optimized regeneration from explants in canola. AgNO3 is a potent inhibitor of ethylene action and ethylene is considered to prevent shoot morphogenesis in vitro (Akasaka-Kennedy et al., 2005). Therefore, AgNO3 is routinely used in Brassica tissue culture (Cardoza and Stewart, 2004), so that Tang et al. (2003) used AgNO3 for this purpose. De Block et al. (1989) reported that AgNO3 is essential for obtaining shoots from hypocotyls of B. napus.
Ululai et al. (2008) observed high frequency of shoot regeneration in some of used cultivars. The absence of AgNO₃ in media severely reduced regeneration frequency in Brassica napus (Phogat et al., 2000). Akasaka-Kennedy et al. (2005) showed that AgNO₃ has a considerable effect on shoot regeneration. The findings of Curtis et al. (2004), Ozden-Tokatti et al. (2005) and Mundhara and Rashid (2006) were consistent with our findings.

Conclusion

In the present study, an efficient regeneration system for some variety of canola cultivars has been developed. This study confirms that the use of NAA with BAP is better compared to medium without NAA for regeneration potential. Of the 7 cultivars tested, all had the ability to regenerate shoots but with different explants. Also, addition of AgNO₃ to medium was found to be a critical factor for shoot regeneration in all genotypes.

References