INDUCTION OF THE EMBRYOGENESIS PROCESS IN ANTHER AND MICROSPORES CULTURES AT THE *LUPINUS ALBUS* SPECIES

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Abstract

Lupine breeding, and vegetable breeding generally, is constrained by the inability of producing double haploid (DH) plants, which would accelerate the selection and release of new varieties. This technology is still in the developmental phase for vegetables, although other major grain crops such as wheat, barley, and canola successfully use DHs on a commercial scale. The most used technique is the anther culture with microspores at the mono nucleate stage which are being isolated from flower buds and grown in vitro. Recently good results had been obtained using the microspore culture technique. The aim of this study is to evaluate the possibilities of obtaining somatic embryos using both of these techniques on the *Lupinus albus* species. We have obtained embryo - like structures on MS culture medium supplied with 2,4 D and BAP in both cases, but we consider that for the microspore culture, this technique is more efficient.

Key words: anther , embryogenesis, microspore culture.

Using the embryogenesis process of the microspores, regenerated plants can be obtained from a single cell, for example microspore. In optimal growing conditions and using some adequate genotypes, the microspores can generate embryos and after their germination will generate new identical plants. This technique is an advantageous one in the breeding process because is more efficient than the classical techniques.

The microspore cultures have been studied by many authors for a range of species such as: barley (Sunderland and Xu, 1982), wheat (Wfi, 1982, 1988; Datta and Wenzel, 1987), rice (Cho and Zapata 1990; Datta et al. 1990).

First attempts in lupine androgenesis were described by Sator et al. (1983), who obtained only callus from anther culture of *Lupinus polyphyllus*. Plantlets regeneration reported for anther culture of *L. polyphyllus* by Sator (1985) and the chromosomes count suggested that they were diploid. Ormerod and Caligari (1994) observed embryo-like structures (ELS) with clearly defined cotyledons and radicals in liquid culture of *Lupinus albus* microspores. Recurrent somatic embryogenesis occurred following internal cleavage within the ELS or on the surface of the ELS. Direct conversion of ELS was not observed. It was difficult to recognize the origin of induced calluses and ELS, because anther cultures contained both haploid microspores and diploid tissues. Bayliss et al. (2004) reported a reproducible method of microspore culture that led to cell division and pro-embryo formation in *L. albus* and *L. angustifolius*. Further development of these multicellular structures or pro-embryos was limited by the rigid outer exine layer. A general protocol of androgenesis described by Wand et al 2000 has the following steps; 1) harvesting the material in the adequate stage for adrogenesis, 2) prior treatment of the fresh material with low temperatures (Gaillard et al 1991, Kasha et al 2001], osmotic stress (Turaev et al 1997), thermal shock (Custers et al 1994).

The aim of this study was the evaluation of the possibilities for obtaining somatic embryos using both of these techniques at the *Lupinus albu*s species using the microspore and the anther culture in MS (Murashige and Skoog) culture medium supplied with 2,4 D and BAP. We tested temperature prior treatments to buds and phytohormonal compounds in the media.

MATERIAL AND METHOD

For the anther and the microspore culture, we used one cultivar of *Lupinus albus* (Medi) cultivated at the Ezareni farm in 2010. From this cultivar there were collected flower buds at different stages of development. The microspore stages were determined by cytological exam.

a) The anther culture

For the anther culture were used flower buds of 8-10 mm size. The flower buds surface was sterilized in 2% sodium hypochlorite for 20 min and then rinsed three times with distillated water.

Anthers were squashed and stained in aceto-carmine and examined in the light microscope to confirm the initial stage of the microspores. Anthers evaluated to be in the right

stage were aseptically excised and placed in 150 mm Petri dishes (25 anthers per dish) with 15 ml of induction medium MS supplemented with 2,4 D and K in different concentrations (0.1 mg/l, 0,5 mg/l and 1mg/l). The dishes were placed in dark at either 25 or 30 ^{o}C.

b) The microspores culture

The flower buds were sterilized 20 min in 2% sodium hypochlorite, rinsed with distillate water and homogenized in MS liquid medium containing 13% sucrose, in a blender. The homogenate was centrifuged 3 times at 1100 rpm for 3 min for the pure microspore preparation. The purified microspores were re-suspended in 1 ml medium MS, without hormones. The inoculums was poured in 150 mm Petri dishes with 15 ml solid MS medium supplemented with different concentrations (0.1 mg/l, 0,5 mg/l and 1 mg/l) of 2,4 D and K. The microspores were incubated in different temperature conditions for 15 days. Some of them were kept continuous at 25^{0}C. Others were kept 3 days at 32^{0}C and then 12 days were placed at 25^{0}C. The last group was maintained continuously at 30^{0}C.

Table 1

Frequency of appearance of the pro embryons structures in the anthere culture

Culture medium (auxine/cit)	Number of repetitions	Anthers	Microspores
MS1 (1/1)	1 2 3	24 22 21	61 73 65
average	2	22,3	56.30
MS 2 (1/0,5)	1 2 3	14 12 11	67 52 49
average	2	12,3	56,00
MS 3 (1/0,1)	1 2 3	10 11 8	55 43 41
average	2	9,6	46.33
MS 4 (0.5/1)	1 2 3	2 3 6	35 47 40
average		3,6	40.66
MS 5 (0,1/1)	1 2 3	0 0 0	15 30 27
average	2		24.00

Table 2

Frequency of appearance of the pro-embryos structures (%) in the microspore culture in different temperature conditions

Genotype	Number of repeats	Temperature continuous 25°C	ContinousTemperature 30° C	Temperature variable 32°C si 25°C
Medie	1 2 3	51 73 45	45 51 42	75 81 68
	average	56.30	46	74,6

RESULTS AND DISCUSSIONS

Determining the optimum developmental stage of anthers to be collected for the androgenesis process was made examining the pollen grains from the anthers of different bud sizes at the optical microscope. It has been observed that buds of 16–20 mm size, with flower petals still inside and with 2 mm anthers size were the most appropriate for anther culture because they contained microspores in the late mono nucleate stage of development. Their cultivation on culture medium led to the appearance of some embryo – like structures after 15 days. Short anthers easily released microspores to the media; however, androgenesis was not observed in these microspores and they died 7 days after the start of the culture. For the long anthers, microspores released to medium were more viable, but microscopic observations indicated the presence of small microspores with dense cytoplasm that did not divide. *Table nr.1* shows that the intensity of the embryogenetical response is higher in the microspore culture than in the anthers culture. It was not influenced by the culture medium. Still the hormonal balance of the medium influences the intensity of the embryo reaction of the microspores. Comparing the two types of hormones used, the auxine might have the higher

influence. The frequency of the pro-embryo structures decreases with the reduction of the 2,4D concentration. It was noticed the total absence of the embryo response in the anther cultures when the 2,4 D concentration decreased to 0,1mg/l. The stimulatory effect of auxines on the microspore embryogenesis was shown in 3-day-pretreated tobacco anthers (Imamura and Harada 1980a). Moreover, in *Hordeum vulgare* L., the addition of ABA to the anther cultures increased the viability and reduced the occurrence of apoptotic characteristics in the microspores (Wang et al. 1999). Because ABA is an inhibitor of mRNA synthesis, it may be possible that treatment with ABA inhibits the synthesis of certain RNAs necessary for gametophyte development thereby blocking gametophyte pathway and thus switching on the sporophytic development (Imamura and Harada 1980a). Prior treatment of anthers with auxines stimulated microspore viability and regeneration efficiency in barley (Van Bergen et al. 1999). On the other hand, increasing the concentration of kinetin associated with a high content of auxine brings both quantitative (increase the microspore frequency with the present internal divisions) and qualitative changes (appearance of bipolar structure). Basically, the auxins could play the role of regulating the development of the microspores from the stage of gametophyte to the stage of embryo, but the further evolution is more efficient in the presence of the citochinines. In conclusion, the culture medium (MS) supplemented with equal amount of auxine and citokinine is the most indicated to induce the embryogenesis process.

In the other experiment it was used the culture of microspores grown on MS medium supplemented with 1 mg / l 2,4 D and 1 mg / l K. They were subjected to different heat treatments: continous 25°C temperature, continuous 30°C temperature and variable temperature of 32 – 25 ^{o}C. Variants mantained at continous temperature did not show notable differences. The increase of the temperature to 30^{0}C determined the decrease of the frequency of appearance of the pro-embryo structures (*Table 2*). In contrast, applying the short temperature shock then the decrease of the temperature to 25^{0}C seems to determine the microspores transition from the embryo stage to the gametophytic one.

Microspores can be induced to become embryogenesis plants, or in excised inflorescences or flower buds, or *in vitro* cultured anthers or isolated microspores (Touraev et al. 1997). In all the cases, conversion to the sporophytic stage can be induced by subjecting microspores to various stresses. On other studies heat shock has been used as a trigger to induce embryogenesis in isolated microspores of various species, like: rapeseed,wheat, tobacco, eggplant etc.

CONCLUSIONS

The results of the study show that the optimum stage of micronuclei development at the *Lupinus albus* species is directly related with the size of the flower buds for the anther and the microspore cultures. On the other hand, increasing the auxins content intensified the embryo response of the microspores in both cases of cultures, but the best results were obtained using the culture medium supplemented with auxins and cytokines at the rate of 1/1.

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