IN VITRO GYNOGENESIS IN RED BEET (*BETA VULGARIS* L): EFFECTS OF OVULE CULTURE CONDITIONS

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ABSTRACT

In this paper the influence of factors affecting gynogenic response of red beet ovules is discussed. The ovule response frequencies were the highest in the following conditions: N₆ (Chu 1975) mineral salts, 0.5 mg/l IAA, 0.2 mg/l BA, 27 or 32°C. The influence of genotype of donor plants was confirmed and it was found that the ovules excised from cultivar plants have a greater gynogenic ability than the ovules of hybrids or inbred lines.

KEY WORDS: *Beta vulgaris*, gynogenesis, haploid, *in vitro* ovule culture.

INTRODUCTION

The process of establishing true breeding lines is a critical step on the way to produce new cultivars. This difficulty mainly occurs when allogamous species are used. Traditionally, plant breeders use the time-consuming processes of inbreeding or backcrossing in order to achieve homozygosity for all the genes. This can be sped up by a production of haploid plants, their diploidization and then multiplication, thus finally the completely homozygous doubled-haploid lines are established. Doubled-haploid (DH) lines were obtained for over 200 species. They can be achieved in several ways. The most common are immature embryo cultures of barley and wheat after interspecific crosses with *Hordeum vulgare* or maize. The next method requires applying the *in vitro* culture of gametes, either microspores (androgenesis) or megaspores (gynogenesis). Potentially, androgenesis can be the most efficient in the production of haploids due to the large number of microspores in anthers. Androgenesis has been applied for many species and a number of cultivars have been released from breeding programs using this method. Gynogenesis is a much less efficient method for haploid production, because of the usually small number of ovules in ovaries. This is why gynogenesis can be used in a few species only where the other methods failed. Until now production of haploids by gynogenic development has been reported in: *Gerbera jamesonii* (Meynet and Sibi 1984), *Allium cepa* (Muren 1989, Keller 1990), *Oryza sativa*, *Nicotiana tabacum*, *Hordeum vulgare*, *Triticum aestivum* and others (Yang and Zhou 1982).

The first results of *in vitro* gynogenesis in sugar beet were announced in 1983 by Bossoutrot and Hosemans (1985) and by Goška (1985). Since then the production of DH sugar beet lines by unfertilized ovule induction has been improved and now is being introduced into breeding programs. The efficiency of gynogenesis is determined by several factors, such as genotype of donor plants, embryo sac stage, cold or heat shock pre-treatment, culture media and culture conditions.

The influence of some of these factors has been studied in red beet, due to the great importance of this vegetable in Poland and the fact, that only few cultivars are available.

MATERIAL AND METHODS

The study of gynogenesis in red beet has been carried out for 3 years. Vernalized red beet roots planted in January in a greenhouse started blooming at the end of March. In May roots were planted in a field where they bloomed at the end of June. Flower buds were collected about two days before anthesis. The buds were kept for 30 s. in 50% alcohol to remove fat, sterilised in 10% chloramine for 15 min and then washed 3 times with sterile distilled water. Unfertilized ovules were excised from the buds in a flow cabinet and placed in flasks containing 40 ml of medium. The ovules were cultured in dark for 2 days and then were moved to a room with 16/8 h fotoperiod.

Plant material.

In this study 15 red beet genotypes of different origin were used. The collection consisted of 4 open-pollinated cultivars, 6 F₁ hybrids and 5 inbred lines (Table 1). Plants of male sterile and maintenance lines were considered as one genotype.

TABLE 1. Genotypes of the donor plants used for the ovule culture.

Cultivars	F ₁ hybrids	Inbred lines
Czerwona Kula	218 x 279	218
Okragły Ciemnoczerwony	279 x 218	260
Crosby	391 x 279	279
Egipski	N 10594	357
	N 10595	391
	N 10596	

Culture media.

Two mineral salt compositions were compared. They were based on MS medium (Murashige and Shoog 1962) and N₆ medium described by Chu (Doctrinal 1990). These media were supplemented with: thiamine 1 mg/l, pyridoxine 0.5 mg/l and nicotinic acid 0.5 mg/l. The influence of two growth regulators at various concentrations was investigated. These were as follows: indole-3-acetic acid (IAA) 0, 0.25, 0.5, 1 mg/l and 6-benzylaminopurine (BA) 0.2, 0.5, 1 mg/l. The final solution contained 6% of sucrose and 0.75% of agar. Prior to autoclaving at 121°C the pH value of the media was adjusted to 5.7-5.8. Some additional media based on MS were also used. In that case IAA was replaced by NAA (α -naphthaleneacetic acid).

Temperature.

For isothermic runs three different temperatures 25, 27 and 32°C were chosen. In polythermic runs the temperature during the initial period of 48 hours was kept at different levels. Thus the following temperature programs were applied: 32°C followed by 27°C and 25°C followed by 32°C.

RESULTS

The first symptoms of the ovule response were noticed in the very beginning of the culture. At that time, the colour of the majority of the ovules changed from white to deep-red, and their diameter almost doubled so their enlargement was clearly visible. During the next 2-3 weeks the majority of the ovules turned brown while the others remained deep-red. The first signs of development of callus or an organised tissue was noticed after 16 days from the start of the culture, but the average time necessary to begin the development was about 4 weeks. Both the brown and deep-red ovules developed further and no difference between them was noticed. This development could proceed in three ways. In the first one 60% of the cultured ovules developed a deep-red callus of a friable and soft structure. 20% of the cultured ovules formed the radicle and then the cotyledons appeared directly out of the ovules. The remaining 20% developed the radicle or a structure of a similar shape; covered rapidly by callus.

Experiment modifiers – influence on ovule response:

Growth regulators

The percentage of ovules responding to different concentrations of growth regulators varied from 1.03% to 4.14%. The differences between these percentages, calculated on various IAA concentrations, were not significant. The same conclusion is true when BA was taken into account. On the basis of IAA – BA interaction (Fig. 1) the combination of 0.5 mg/l IAA and 0.2 mg/l BA was chosen as the best for gynogenesis and this combination was used in all further experiments. The use of NAA instead of IAA resulted in a much lower gynogenic response.

Mineral salts

The ovule response frequencies on MS or N₆ medium were different in the consecutive years. In the first year the difference was not significant. In the next year significantly better results were obtained when N₆ medium was used. These differences were noticed for both greenhouse and field-grown plants (Table 2).

Fig. 1. The effect of IAA-BA interaction on the percentage of responding ovules.

TABLE 2. The effect of mineral salts composition on the ovule response frequencies in different seasons.

Season		MS (%)	N ₆ (%)
1 st year	greenhouse	8.57	5.00
	field	2.86	3.33
	mean for 1 st year ¹	5.71	4.14
2 nd year	greenhouse	8.83	12.17
	field	2.38	4.44
	mean for 2 nd year ²	5.61	8.30

¹differences not significant

²differences significant at 1.8% level

Temperature

Temperature was an important factor in gynogenesis in red beet. The preliminary experiment concerning the increase of temperature from 25°C to 27°C resulted in doubling of the number of responding ovules. This result was confirmed in an experiment where several temperatures were tested. There was a great difference in response frequencies of the ovules grown in 25°C and at higher temperatures (Fig. 2). This dependence was observed for all media used and both donor plant growth conditions. There were no significant differences when temperatures of 27 or 32°C were applied. The ovule response was affected by the temperature during the first 48h of culture. The culture in 25°C and then moved to 32°C reacted similarly to the culture kept in 25°C all the time (Table 3).

Fig. 2. The effect of temperature on the percentage of responding ovules.

TABLE 3. The comparison of isothermal and polythermal treatment of the culture.

Temperature (°C)	% of responding ovules
25	4.00 c
25 for 48h followed by 32	2.00 c
27	12.67 a
32	11.33 ab
32 for 48h followed by 27	7.33 b

differences significant at 5% level

Genotype

There is a great variation in gynogenic ability depending on the genotype of the donor plants (Table 4). In all experiments cultivars were characterised by the best gynogenic ability. The number of developing ovules originated from cultivars was a few times higher than that from hybrids or inbred lines. One hybrid and one inbred line did not respond to gynogenic induction at all. The percentage of the developing ovules for the cultivars was 3.9%, and for hybrids and inbred lines 0.84% and 0.29% respectively. This percentage was doubled when the cultivar ovules were cultured at "optimal" conditions (e.g. N₆ medium at 27°C).

TABLE 4. The effect of donor plant genotype on the ovule response frequencies.

Genotype	Mean for 3 years	Mean for N ₆ medium at 27°C
Czerwona Kula	4.14	7.25
Okrągły Ciemnoczerwony	3.65	7.96
Crosby	4.56	11.24
Egipski	3.48	5.00
218 x 279	1.49	1.66
279 x 218	0.57	0
391 x 279	0	0
N 10594	2.86 *	2.50
N 10595	1.33 *	0
N 10596	1.66 *	1.66
218	0.24	0
260	0	0
279	0.34	0
357	0.38 **	0
391	0.38	0

* The mean value calculated for 1 year

** The mean value calculated for 2 years

Season

In a three-year study a progress in induction frequencies was noticed. This is due to the improvement of culture conditions. There were no differences in the number of developing ovules between spring and summer for 2 consecutive years. Difference occurred in the last experimental year. The difference was noticed in the ovules originated from the cultivar plants: the greenhouse conditions were better than the field ones (Table 5).

TABLE 5. Comparison of the ovules response frequencies for the donor plants growth conditions in the consecutive years.

Season	1 st year	2 nd year	3 th year
greenhouse	1.77	3.28	10.50
field	1.20	3.52	3.41
Mean for 3 cultivars on MS medium			
greenhouse	2.93	4.10	8.83
field	0.88	3.38	2.38

DISCUSSION

To my knowledge, there are no reports about gynogenesis in red beet. All the papers published concern sugar beet. Sugar beet and red beet belong to the same species and thus some similarity can be expected.

In ovule or ovary culture haploid plants can arise when they originate from haploid cells. The embryo sac in beet consists of 7-10 haploid cells: the egg cell, the persistent synergid, the degenerated synergid and 4-7 antipodals (Bruun 1987). A cytological study of gynogenesis was reported by Ferrant and Bouharmont (1994) who state that development of the egg cell only is observed. This development can occur as a direct organogenesis (Van Geyt et al 1987) or as callus formation (Hosemans and Bossoutrot 1983). It was proved that both organogenic and embryogenic callus can be haploid; thus the production of haploid plants is the matter of further organogenesis. Before fertilization the ovule is about 1 mm in diameter, and the embryo sac is mature and well developed (Artschwager 1927). As the embryo sac of sugar beet is mature before anthesis (Brunn 1987, Ferrant and Bouharmont 1994), the ovules excised 2 days before that are fully developed.

Improvement of culture conditions and medium composition can have an essential influence on gynogenic ability. A major effect was observed when mineral salt composition in media was changed. The medium most often used for gynogenesis in sugar beet is MS. The advantage of using N₆ medium instead of MS has been shown in this paper. The insignificant differences between these media in the first year of experiment are probably due to the influence of temperature, the most important culture condition factor. These results are in agreement with Doctrinal et al (1990). The main difference in the media mineral composition concerns the total nitrogen content. In comparison to MS medium N₆ contains half the amount of nitrogen (35 mM) and has a higher inorganic NO₃ : NO₄ ratio (80:20) while MS 65:35. The effect of nitrogen content is discussed by Lörz (Opsahl-Ferstad 1994) who found that 20-35 mM of total nitrogen was optimal for development of barley microspores. Some authors report that a high inorganic NO₃ : NO₄ ratio can increase microspore sensitivity to auxin and therefore this ratio seems to be important.

A small effect of growth regulators was observed in this study. This is consistent with Lux et al (1990) who stated a minor importance of auxin content, and with Doctrinal et al (1989) who reported that changing of IAA content did not bring about any improvement. However, the influence of different BA concentrations on the ovule response has not been corroborated by the results presented in this paper.

As in other crops the genotype of red beet donor plants was found to be the most important factor determining success of

the ovule culture. The ovule response is very strongly dependent on the genotype and varies from 0-17% (Yang and Zhou 1982, Van Geyt et al 1987, Galatowitsch and Smith 1989, Wang et al 1989). In some particular genotypes even 30% of ovules responded (Wang et al 1987). These reports indicate that this process is controlled by several genes. In this study an essential difference between cultivars and hybrids or inbred lines was found. The higher gynogenic ability of cultivars can be caused by their genetic variability. Inbreeding and the related to it depression may play an important role in gynogenic ability, too. The ovules of inbred lines were much smaller than the ovules of cultivars.

The presented results for the cultivars indicate that the gynogenic response depends on the conditions the donor plants grow under. A similar effect was noticed by Doctrinal et al (1989) and Lux et al (1990), who tested gynogenic response of plants grown in a greenhouse or in a field in different periods of the year. In contrary to their results March/April seems to be a better term for gynogenesis than July. In this study both the greenhouse and field conditions were applied. It is difficult to determine if the gynogenic response is more affected by season or growth conditions. The lack of significant differences between seasons when ovules of all of genotypes were compared is due to a large number of inbred lines and hybrids without any gynogenic response.

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GYNOGENEZA IN VITRO U BURAKA ĆWIKŁOWEGO (*BETA VULGARIS* L.): WPŁYW WARUNKÓW PROWADZENIA KULTUR NA ROZWÓJ ZAŁĄŻKÓW

STRESZCZENIE

Prezentowana praca opisuje wpływ czynników genetycznych, termicznych i chemicznych na rozwój gyno-geniczny z załączków buraka ćwikłowego. Najlepsze warunki do rozwoju załączków uzyskano, gdy zastosowano pożywkę o składzie mineralnym takim jak w pożywce N₆ (Chu 1975) z dodatkiem 0.5 mg/l IAA i 0.2 mg/l BA, a kultury trzymano w 27 lub 32°C. Potwierdzono również bardzo silny wpływ genotypu roślin donorowych i wykazano, że odmiany mają większą zdolność do gynogenezy aniżeli mieszańce F₁ i linie wsobne.

SŁOWA KLUCZOWE: *Beta vulgaris*, burak ćwikłowy, gynogeneza, haploid, kultury załączków in vitro.