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THE COMPOSITION OF CULTURE MEDIA FOR INDUCTION OF CALLUSOGENESIS IN THE ANTHER CULTURE OF SUGAR BEET

In the article the results on optimization of composition culture media are presented on macro-and microelements, carbohydrates, vitamins, amino acids, plant grows regulators callus for induction of callusogenesis in the anther culture of sugar beet. Influence of different classes of plant growth regulators on the process of induction of callusogenesis and the ways of propagation of androgenic calluses are shown in the anther culture of sugar beet auxins (2,4-D), cytokinins (6-BAP and kinetin) and ABA, aminoacids is investigated.

Keywords: *androgenesis; culture in vitro; sugar beet; anther; callus*

Introduction. Composition of culture media, intended to induce the formation of morphogenic callus culture of isolated anthers as intermediaries for obtaining haploid plants and dihaployid is a main element that determines the success in the development of the indirect androgenesis.

Presently known are methods of direct and indirect androgenesis which are designed for different cultures, including for agricultural use: carrots [12], tomatoes, cabbage, onions [13], maize [7], potatoes [5], wheat [3] rape [4] and others. As the requirements of tissues, explants of different plant species to food sources of vitamins and growth regulators are widely divergent, the authors of these techniques have been optimized compositions of culture media that it is specific to anther tissue and pollen of these plants [3, 4, 5, 7, 12 13]. It should be noted that despite the general principles for the creation of formulations composition of culture media [10], selection of media elements in most cases is an empirical, and not only contributes to the scheme of experiments and experimental work in general, but also affects the results, which in most cases are quite low.

With respect to sugar beet, there are literature sources that demonstrate not only their ability to form callus from explants isolated from different parts of the plant such as stems, leaves, unfertilized seed primordia [2, 14], but the high morphogenic activity of various tissues and organs of sugar beet [11]. As for androgenic anthers callus of sugar beet such data are not known.

Analysis of the literature and pooling the requirements to the culture media designed for callusogenesis induction in anther culture of different cultures [3, 4, 5, 7, 12, 13] showed that the most popular for modification environment is one by Murasihe-Skoog (MS) [6 10], which contains a balanced amount of nutrients and Hamborha one [6, 10], where the total salt concentration is lower than among MS, but there are more nitrate nitrogen than ammonic , both in the original prescription and modified. For further improvement, medias by Genovese, Pirika, Nicha and Night and others are using. Intended for the cultivation of tobacco and cereal anther induction callus [3, 4, 6, 10]. A common feature of all modified environments being used for induction callusogenesis, is reduced concentration of macronutrients. Their optimal concentration as well as the micronutrients, should be established experimentally. As a carbon source for most plants sucrose is used [10]. Glutamine is the nutrients amino acid that is used in the media to callusogenesis but recently quite extensively studied the role of other amino acids in the processes of morphogenesis [4, 7, 8].

Regarding growth regulators, it should be noted that the positive effect occurs mainly when using 2,4-D as a major growth regulator that stimulates cell division and tissue explants dedifferencetion and promotes proliferation of calluses, but in the presence of one or two different cytokinins, particularly 6-BAP and kinetin [9]. There is an evidence on the positive role of ABA in growth inhibitor initiation of callusogenesis [3, 10].

The content of vitamins that are essential components of culture media, was also subject to modification, including the majority of authors increased content of thiamine and nicotinic acid [10].

Research goal. To determine the role of the main elements and elaborate composition of culture media, intended to induce callusogenesis in anther culture of sugar beet.

Research objectives:

1. To analyze the role of the main elements that make up the culture media designed for callusogenesis induction in anther culture of sugar beet.
2. To determine the effect of different classes of growth regulators on the induction process of callusogenesis in anther culture of sugar beet.
3. To optimize culture media on the content of macro-and micronutrients, carbohydrates, vitamins, amino acids, growth regulators to induce callusogenesis in anther culture of sugar beet.

Materials and methods. Experiments were carried out at the Institute of Bioenergy Crops and Sugar Beet during 2012-2014. In studies as selective material we used anther of tetraploid and diploid pollinator of sugar beets, which grew under the conditions of the fields both at Bila Tserkva and Yaltushky Experimental Breeding Station. We used following sugar beet genotypes: 3184K1, 3184K6, 3184K10, K12 3184, 3189K3, 3189 K10, 3189K11, 3189K12, 1257K1 - K15, 1258K1 - 1258K12, 4HMM 1 - 4HMM-8.

Sterilization, pre-processing explants, inoculation and cultivation of anthers we performed using the general schemes and methods for other cultures [7, 10] and adapted to work with anthers and pollen of sugar beet culture *in vitro*. For pre-processing plant material (Stage 1) low-temperature stress was applied: shoots with buds of sugar beet seed bearers were kept in the refrigerator at 4-10°C, with 16-hour lighting (1,000-2,000 lux within 7-30 days . To sterilize the plant material (Stage 2) used were solutions of ethanol, hypoclorite natrium, hydrogen peroxide. The anther was isolated from sterilized buds and inoculated into culture media (3rd stage). It was cultured in the dark at a temperature of 26-32^{0}C and relative humidity of 50-70% to proliferate callus (Step 4).

To initiate callusogenesis developed and produced were several series of culture media, which differed on the content of macroelements, aminoacids, vitamins, growth regulators, carbohydrates. The prescriptions are given in Table 1.

As a basic medium we took the medium by Murasihe - Skoog [10] with full and reduced 1/2 macronutrients with vitamins by prescription Hamborha [10], with the addition of ascorbic acid 1.0 mg/l, and the amino acid glutamine 500 mg/l, sucrose 40 g/l. Modification of the environment was carried out by the addition to the base medium of the following components: amino acids (aspartic acid 30.0-50.0 mg/l, arginine 2.0-250.0 mg/l, proline 1.0-5.0 mg/l, hydroxyproline, tyrosine, glycine at a dose of 2.0-5.0 mg/l), growth regulators (2,4-D 2.0 mg/l, 6-BAP 0.6-4.0 mg/l, ABA 0.03-0.3 mg/l, kinetin 2.0 mg/l, NOC 0.1-0.5 mg/l), vitamins (ascorbic acid 5.0 mg/l, folic acid 0.5 mg/l.)

Calculations and observations in the experiments were carried out taking into account suggestions by Satarova [7].

In the experiments, we determined: total number of anthers, which were planted in each medium; number of anthers that reveal morphogenic activity and number calluses as a percentage of the number of anthers planted for each environment. The experiments were repeated six to eight times. Statistical analysis of the data was performed using the software Statistics 5.1.

Results and discussion. The results on optimization of the culture media to induce callusogenesis in anther culture of sugar beet by groups of components such as macronutrients, amino acids, vitamins, growth regulators, carbohydrates are shown in Table 1.

Table 1

The composition of culture media for induction callusogenesis in anther culture of sugar beet

Components of media	Options of media													
	СЕ	МСп	П1	П2	П3	П4	П5	П10	П11	П12	П15	П8	П9	П7
Macronutrients (MC)	1	1	1/2	1/2	1/2	1 1/2	1/2	1 1/2	1 1/2	1 1/2	1/2	1 1/2	1 1/2	1/2
Micronutrients (MC)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Aminoacids														
Glutamine	12.5	500	500	500	500	500	500	500	500	500	500	500	500	500
Glycine	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Arginine	-	3.0	3.0	250	3.0	250	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Asparagine	-	30	30	100	30	30	100	30	30	30	30	30	30	30
Proline	-	-	-	-	-	5.0	5.0	-	-	-	-	-	-	-
Tryptophan	-	-	-	-	-	-		-	-	-	-	-	-	-
Growth regulators														
2,4-D	-	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
6- BAP	0.1	0.6	0.6	0.6	0.6	0.6	0.6	1.0	4.0	0.6	0.6	0.6	0.6	0.6
ABA	-	-	-	-	-	-	-	-	-	-	-	-	-	0.03-0.3
Kinetin	-	-	-	-	-	-	-	-	-	2.0	-	-	-	-
NAA	0.2	-	-	-	-	-	-	0.5	0.1	0.1	-	-	-	-
GA	0.25	-	-	-	-	-	-	-	-	-	-	-	-	-
Vitamins														
B1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0,1	0.1	0.1	0.1	0.1
B6	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
PP	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Вит.С	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.0	1.0	1.0	1.0
Folic acid	-	-	-	-	-	-	-	-	-	-	0.5	-	-	-
Othes organic impurities														
Sucrose (g)	15	40	40	40	40	40	40	40	40	40	40	90-120	-	40
Maltose (g)	-	-	-	-	-	-	-	-	-	-	-	-	100	-
Mesoinosite	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Agar	9	9	9	9	9	9	9	9	9	9	9	9	9	9

The results showed that almost all nutrient media observed the initiation of tumors (an unusual structure in the form of tubes (stamen filament) with a thickening at the tip, which developed from the tissues of the central part of the anther (Fig. 1) and primary callus. Callus initiated by various tissues, tips of stamen filaments (Fig. 2) and directly by the anther tissues (Fig. 3).

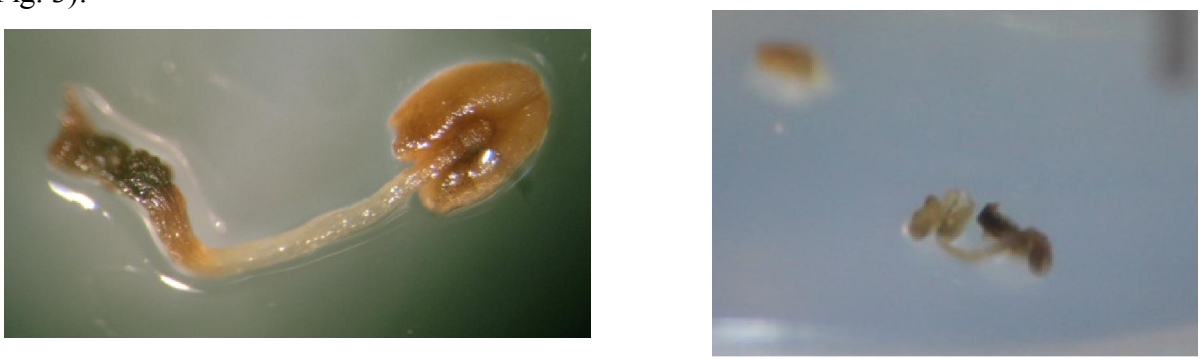

Fig. 1. Development initiating calluses tissue structures on the tips of stamen filament in the anther of sugar beet

Fig. 2. Proliferation of callus stamen filament in the anther of sugar beet

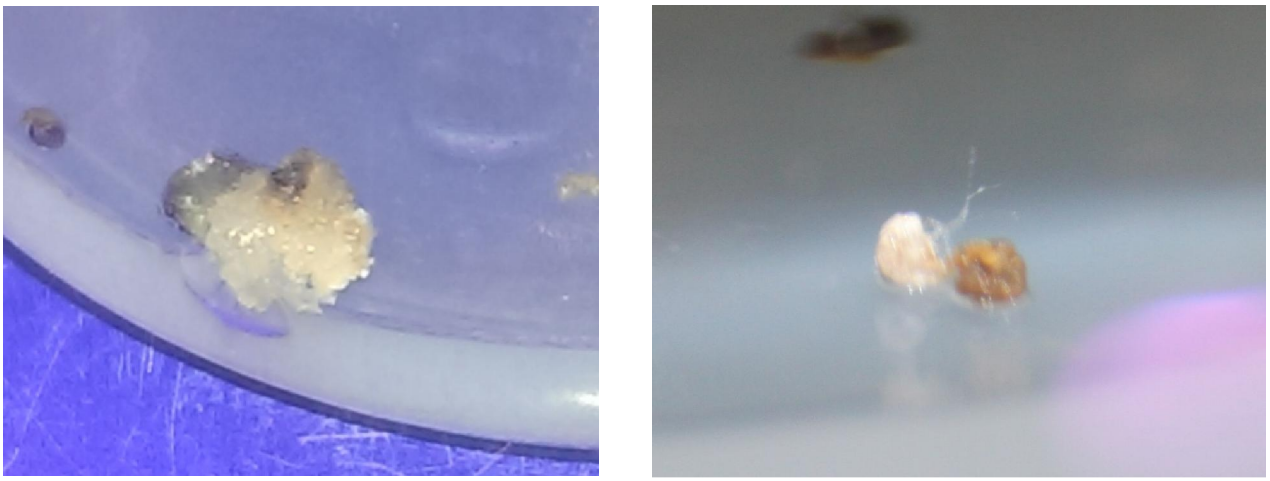

Fig. 3. Varieties of primary calluses in the anther of sugar beet

The first tumors appeared in 6-7 days from the start of cultivation. The number of tumors was 1. 63-7.70 % of the anthers planted on modified culture media (Table 2).

Table 2

The influence of the composition of the culture medium at the initiation of tumors in anther culture of sugar beet

Options of mediums	Number of planted anthers, pcs.	Number of active morphogenic anther,%	Number of received callus, %
CE	195	5.12 ±0.41	-
МСП	257	3.50±0.23	-
П1	177	7.34±0.57	0.56±0.27
П2	248	7.66±0.63	-
П3	224	1.78±0.14	-
П4	254	4.72±0.37	1.18±0.54
П5	245	1.63±0.12	-
П7	I83	7.10±0.64	0.54±0.21
П8	293	5.80±0.48	0.68±0.37
П9	116	7.70±0.35	0.86±0.44
П10	246	2.43±0.19	-
П11	I35	-	-
П12	I47	4.76±0.31	0.68±0.31
П15	94	3.10±0.23	-

The number of primary calluses was significantly lower compared to the total number of tumors - 0,54-1,18% of planted anthers. The most effective for the initiation process was callusogenesis environment П4, which had a membership base growth regulators 2,4-D (2.0 mg/l) and 6-BAP (0.6 mg/l), and was supplemented with the amino acid proline (5.0 mg/l).

In most environments as the cytokinin 6-BAP was used (0.6 mg/l). Addition of the environment kinetin (2.0 mg/l) - П12 environment contributed callusogenesis that is the formation of tissues callus from anthers.

The introduction of the inhibitor of growth media ABA (medium П7) at concentrations recommended for culture media (0.03 - 0.30 mg /l) stimulated morphogenesis. But callus proliferation was observed only in the cultivation of sugar beet anthers on media with ABA 0.3 mg/l.

It is well known that the growth of a number of isolated plant tissues is activated when introduced into the environment of certain L-aminoacids, often glycine [8], or mixtures thereof, such as casein hydrolyzate [6]. However, the need for these substances for each is different. Thus, the addition proline, oxyproline, glutamine to culture medium for culturing anthers of cereals increases the frequency of formation of androgenic structures [4], in particular, the addition of proline 6 times oxyproline more than 4 times and glutamine 5 times as much.

As a result of our studies it was found that the use of proline in the same amount of 5.0 mg /l contributed to the increase obtained calluses twice (medium П4), while increasing the dose of arginine from 3 mg /l to 250 mg/l (medium П2) and the use of tryptophan (medium П5) did not affect the process of callusogenesis.

Increase in the amount of sucrose from 30 g/l to 60 g/l and even 120 g/l in nutrient composition (П8 environment) does not significantly influence the number of tumors. Replacing sucrose to maltose (medium П9) also did not affect the number of callus formed, but contributed to a significant increase in the size of planted anthers. We found that out of 43 genotypes studied, morphogenic were almost all but callusogenic only 13. The most calusogenic activity was observed in genotypes 3184 K10, 1257 K3. Observations showed that the primary calluses were mostly white or translucent, have a homogeneous structure but different texture (plump, firm). After transferring the flasks with callus in terms of culture room with lighting for 18 hours calluses acquired more saturated colours and were increasing in size. These primary callus were used for further research processes on androgenic culture in vitro.

Conclusions.

1. Determined was the effect of different classes of growth regulators such as auxins (2,4-D), cytokinin (6-BAP and kinetynu) and ABA on the induction process callusogenesis in the anther culture of sugar beet. Established that there is a positive effect mainly when using 2,4-D as a major growth regulator that promotes cell proliferation callus, but in the presence of cytokinin, including 6-BAP and kinetin. At application of ABA callus proliferation was observed only at doses of 0.3 mg/l.

2. Optimized were compositions of culture media on the content of macro-and micronutrients, carbohydrates, vitamins, amino acids, growth regulators to induce callusogenesis in the anther culture of sugar beet and received androgen callus.

3. The most effective to initiate processes of callusogenesis established to be modified Murasihe-Skoog medium, which had basic growth regulators 2,4-D (2.0 mg/l) and 6-BAP (0.6 mg/l), and was supplemented with the amino acid proline (5.0 mg/l).

References

1. Белинская Е.В. Влияние элементов технологии гаплоидной индукции на проявление генотипических особенностей морфогенеза в культуре пыльников in vitro ярового ячменя / Е.В. Белинская // Цитология и генетика. – 2010. – Т. 44. – № 2. – С. 38-44.

2. Белоус В.Е. Биотехнологические методы в селекции сахарной свеклы/ В.Е. Белоус, Н.И. Ильенко, В.И. Редько. – М.: Агропромиздат, 1989. – С. 32-41.

3. Горбунова В.Ю. Андрогенез in vitro у яровой мягкой пшеницы: дис... д-ра наук: 03.00.15 / В.Ю. Горбунова. – Уфа, 2000. – 290 с.

4. Ігнатова С.О. Біотехногічні основи одержання гаплоїдів, віддалених гібридів і соматичних регенерантів зернових і бобових культур в різних системах in vitro: автореф.дис... д-ра наук: 03.00.20/ Ігнатова С.О. – Ялта, 2004. – 48с.

5. Маруненко И.М. Каллусогенез и эмбриогенез в культуре пыльников картофеля / Маруненко И.М. , Кучко А.А. / Цитология и генетика. 1989. –Т. 23, № 3. – С. 48-51.

6. Мусієнко М.М. Біотехнологія рослин: навч. посібник / М.М. Мусієнко, О.О. Панюта. – К.: ВПЦ «Київський університет», 2005.– 114 с.

7. Сатарова Т.Н. Андрогенез та ембріокультура у кукурудзи in vitro: дис. д-ра біол. наук: 03.00.20 / Т.Н. Сатарова. – К., 2002. – 537 с.

8. Сатина Т. Г. Влияние глицина на андрогенез пыльников in vitro ярового рапса / А.А. Муравлев. – Л., ВНИИМК, 2009. – С. 192-195.

9. Полевой В.В. Фитогормоны / В.В. Полевой. – Л.: Изд. Ленингр. ун-та, 1982. – 248 с.

10. Кушнір Г.П. Мікроклональне розмноження рослин / Г.П. Кушнір, В.В. Сарнацька. – К., 2005. – 270 с.

11. Подвигина О.А. Теоретическое обоснование и приемы использования методов биотехнологии в селекции сахарной свеклы : дис. ... д-ра с.-х. наук: 06.01.05 / О.А. Подвигина. – Воронеж, 2003. – 280 с.

12. Тюкавин Г.Б. Биотехнологические основы селекционной технологии моркови: Daucus carota L.: дис…д -ра биол. наук: 03.00.23 / Г.Б. Тюкавин. – М., 2007. – 539 с.

13. Шмыкова Н.А. Разработка системы биотехнологических методов, направленных на ускорение селекционного процесса овощных культур: Дис. ..д-ра.биол. наук: 03.00.23 / Н.А. Шмыкова. – М., 2006. – 365 с.

14. Sanders J.W. Shoot regeneration from hormone-autonomous callus and adventitious buds in sugar beet (Beta vulgaris L.) / J.W. Sanders // Plant Sci. Lett. – 1984. – 34. – P. 219-223.

Аннотація

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Живильні середовища для індукції калусогенезу в культурі пиляків цукрових буряків

В статті представлені результати оптимізації складу живильних середовищ за вмістом макро- і мікроелементів, вуглеводів, вітамінів, амінокислот, регуляторів росту, що використовують для індукції калусогенезу в культурі пиляків цукрових буряків. Досліджено вплив різних класів регуляторів росту – ауксинів (2,4-Д), цитокінінів (6-БАП та кінетину) та АБК, амінокислот на процес індукції калусогенезу та показані шляхи отримання андрогенних калусів в культурі пиляків цукрових буряків.

Ключові слова*: андрогенез, культура in vitro, цукрові буряки, пиляк, калус*

Аннотация

Гонтаренко С.М., Герасименко А.Н.

Питательные среды для индукции калусогенеза в культуре пыльников сахарной свеклы

В статье представлены результаты оптимизации состава питательных сред по содержанию макро- и микроэлементов, углеводов, витаминов, аминокислот, регуляторов роста для индукции каллусогенеза в культуре пыльников сахарной свеклы. Исследовано влияние разных классов регуляторов роста – ауксинов (2,4-Д), цитокининов (6-БАП, кинетина) и АБК, аминокислот на процесс индукции каллусогенеза и показаны пути получения андрогенных каллусов в культуре пыльников сахарной свеклы.

Ключевые слова: *андрогенез, культура in vitro, сахарная свекла, пыльник, каллус*