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INDUCTION OF SOMATIC EMBRYOGENESIS IN *Astrophytum asterias* (Zucc.) Lem. IN THE ASPECT OF LIGHT CONDITIONS AND AUXIN 2,4-D CONCENTRATIONS

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Abstract. *Astrophytum asterias* (Zucc.) Lem. is a cactus which is among those most desired by producers and collectors all across the world and, at the same time, a species threatened with extinction in the natural environment. Micropropagation techniques can be helpful both in terms of its *ex situ* protection and its popularisation on the market, thus satisfying the needs of cacti breeders and collectors. Somatic embryogenesis is the most effective method of multiplication and it involves the formation of somatic embryos from vegetative cells. The medium, light conditions and type of explant demonstrate the key effect on its efficiency. Auxin 2,4-D (2,4-Dichlorophenoxyacetic acid) is most frequently applied to embryogenesis induction. In the present study we determined the effect of its concentration and light conditions on the efficiency of *Astrophytum asterias* somatic embryogenesis. Seeds were placed on the modified MS medium with a reduced content of macronutrients and sucrose ½MS (pH 5.7 – before autoclaving). All the *in vitro* cultures were incubated in the growth room (24 ± 2°C, 16 h light/8 h dark photoperiod, the intensity of quantum irradiation: 24.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After 14 days 70% of the seeds were produced of seedlings. To regenerate somatic embryos, halves of green seedlings were placed on the modified MS medium with auxin 2,4-D added at different concentrations: 5; 7 and 10 $mg \cdot dm^{-3}$, the MS0 medium without growth regulators was our control. To verify the effect of light conditions, half of explants were incubated in the light, and half in the dark. After 10 weeks of culture, the regenerated embryos were isolated, counted and measured. They were produced on all the media types, in both light conditions. The present research confirmed a positive effect of 2,4-D and light on the number of explants forming embryoid structures and on the number of regenerating embryos. The most number of embryos per 1 explant (1.8) were obtained on the MS7 medium (7 $mg \cdot dm^{-3}$ 2,4-D) in the light conditions.

Key words: *Astrophytum asterias*, somatic embryos, cactus, micropropagation

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INTRODUCTION

Astrophytum asterias (Zucc.) Lem. is a small spineless cactus (150 × 100 mm), dome-like in shape. It usually has 8 flat ribs, each with a centrally distributed strip of woolly areoles. In natural sites the species occurs in Mexico and Texas. It is important as an ornamental plant, greatly desired in collections [U.S. Fish and Wildlife Service 2003, Martinez-Avalos et al. 2007]. The vegetative reproduction of *A. asterias* was not finally noted under natural conditions, due to a specific mechanism of seed sowing which can fall and germinate right on the mother plant surface [U.S. Fish and Wildlife Service 2003]. Thus, the reproduction of this species gets limited to generative methods [Fleischer and Schutz 1986]. Besides, due to the progressing devastation of the natural habitat, plus the species displacement by competitive grasses, introduced for cattle and goat grazing and finally trampling down, in 1993 it was taken under protection [U.S. Fish and Wildlife Service 2003].

To protect *A. asterias*, one should develop an effective method of its reproduction. Mass propagation accomplished in a short time and at competitive costs, based on tissue cultures, is a promising alternative [Giusti et al. 2002]. The most efficient micropropagation methods include somatic embryogenesis. This method allows for stimulating somatic cells of explants to develop into embryoid structures, referred to as somatic embryos or embryoids, which, besides the mass production, are applied to gene resources protection and in breeding processes at the stage of changed genotypes regeneration [McGranahan et al. 1990, Martinez-Palacios et al. 2003].

The present research followed the preliminary study which verified the effect of lower concentrations of 2,4-D (from 0 to 5 $mg \cdot dm^{-3}$) exposed to light and dark conditions in cactus *Astrophytum asterias* (Zucc.) Lem. Bearing in mind getting the best results at the highest auxin concentration applied, the range of concentration was widened towards higher values.

The present research is the first published report on the induction of the formation of somatic embryos and the effect of auxin 2,4-D and light conditions on the process of somatic embryogenesis in cactus *Astrophytum asterias* (Zucc.) Lem.

MATERIALS AND METHODS

The seedlings of *A. asterias* produced from the seeds originateds from glasshouse cultivation of the Licznerskis' Horticulture Farm at Jarużyn Kolonia in the vicinity of Bydgoszcz were our plant material. The experiment was based on two stages; the first one, which took 14 days, involved sowing sterile seeds on the medium prepared earlier, to produce seedlings the fragments of which at the second stage were placed onto media initiating the process of somatic embryogenesis. Having completed the experiment, the somatic embryos were isolated and exposed to observations under the binocular.

For seeds germination, a modified Murashige and Skoog medium [1962] with half-reduced amount of macronutrients (½MS) and the concentration of sucrose lowered to 20 $g \cdot dm^{-3}$, with no growth regulators, solidified with agar (8 $g \cdot dm^{-3}$, Duchefa) and pH at the level of 5.7 before autoclaving, was applied.

Sterilization of 300 seeds involved rinsing under running water and then in sterile water with a drop of detergent. Next the seeds were surface disinfected for 1–2 s with 70% ethanol and immersed for 15 minutes in the adequate sterilizing agent (1.58% sodium hypochlorite). Then the explants were rinsed 3× in sterile distilled water and the seeds were dried on sterile filter paper and placed on the surface of the medium (1 seed / 350 ml-jar).

In vitro cultures were placed in the growth room under controlled light and temperature conditions. The temperature in the room fell within 24°C ± 2°C.

The day length was 16 hours. The jars, with *in vitro* cultures were exposed to the light of TLD54 fluorescent lamps provided by Philips emitting daylight of an average intensity of quantum irradiation of 24.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

The second stage took 10 weeks. The somatic embryogenesis inducing media contained: macro- and micronutrients according to the modified MS medium [Murashige and Skoog 1962] with 3% (w/v) concentration of sucrose, 100 $mg \cdot dm^{-3}$ myo-inositol, 0.5 $mg \cdot dm^{-3}$ nicotinic acid, 0.5 $mg \cdot dm^{-3}$ pyridoxine, 0.1 $mg \cdot dm^{-3}$ thiamine, 2 $mg \cdot dm^{-3}$ glycine and additional: 330 $mg \cdot dm^{-3}$ $CaCl_2 \cdot 6H_2O$, 13.9 $mg \cdot dm^{-3}$ $FeSO_4 \cdot 7H_2O$ and 20.6 $mg \cdot dm^{-3}$ $Na_2EDTA \cdot 2H_2O$ added, solidified with ultra-pure agar (Purified LAB – AGAR^{TM} provided by Biocorp) at the concentration of 12 $g \cdot dm^{-3}$ with auxins added and pH at the level of 5.7 before autoclaving. Four kinds of them were prepared, varying in the concentration of 2,4-Dichlorophenoxyacetic acid (2,4-D). They contained, respectively: 0 $mg \cdot dm^{-3}$ (the control), 5 $mg \cdot dm^{-3}$, 7 $mg \cdot dm^{-3}$ and 10 $mg \cdot dm^{-3}$ 2,4-D (marked with the following symbols MS0, MS5, MS7, MS10).

The randomly selected sterile seedlings produced at the first stage, similar in terms of colour and size, were cut in the laminar chamber, along the longitudinal axis and the radicle was removed. The halves were placed horizontally, with the cutting surface upwards, 5 into each 350 ml jar. Such a pattern constituted a single replication. The jars with seedling halves were divided into two groups with 6 replicates for each of the four medium types. The first one was placed directly on the shelves in the growth room under the same conditions as at the first stage. The other 24 jars were isolated from the access of light by getting wrapped with a layer of aluminium foil and placed under the same temperature conditions in the growth room.

After 10 weeks all the somatic embryos produced were isolated, counted and measured and their development stage was identified with the use of MS-2TRI binocular. The results were statistically verified with the Student's t-test (significance level of $\alpha = 0.05$).

RESULTS

Sterilization was effective for 83.33% of the inoculated seeds. Germination started after 5 days and accounted for 70%. For the second stage randomly selected, green seedlings, typical for the species were used.

A significant effect of auxin 2,4-D and light conditions was demonstrated on the induction of somatic embryogenesis in *A. asterias* (tab. 1). The number of explants regenerating embryos was higher on the media with auxin 2,4-D added than on the control

Table 1. Number of secondary explants of *Astrophytum asterias* (Zucc.) Lem. forming somatic embryos, adventitious shoots and roots depending on the medium composition and light conditions

Tabela 1. Liczba eksplantatów wtórnych u *Astrophytum asterias* (Zucc.) Lem. regenerujących zarodki somatyczne, pędy przybyszowe i korzenie, w zależności od składu pożywki i warunków świetlnych

		Number of explants Total Liczba eksplantatów Ogółem	Explants – Eksplantaty					
			forming embryos regenerujące zarodki		forming roots regenerujące korzenie		forming shoots regenerujące pędy	
			number liczba	%	number liczba	%	numer liczba	%
Medium Pożywka	MS0	60	9 b*	15.0	39 a	65.0	6 a	10
	MS5	60	19 a	34.5	0 b	0.0	0 b	0
	MS7	60	24 a	43.6	0 b	0.0	0 b	0
	MS10	60	24 a	43.6	0 b	0.0	0 b	0
Light conditions Warunki świetlne	light światło	120	48 a	40.0	19 a	15.8	4 a	3.3
	darkness ciemność	120	28 b	26.7	20 a	19.0	2 a	1.9

*means in columns marked with the same letter do not differ significantly at $\alpha = 0.05$

*średnie w kolumnach oznaczone tymi samymi literami alfabetu nie różnią się istotnie przy poziomie istotności $\alpha = 0,05$

Table 2. Number of regenerated somatic embryos of *Astrophytum asterias* (Zucc.) Lem. on different media and in different light conditions

Tabela 2. Liczba zregenerowanych zarodków somatycznych u *Astrophytum asterias* (Zucc.) Lem. na różnych pożywkach i w różnych warunkach świetlnych

Light conditions Warunki świetlne	Medium Pożywka	Somatic embryos – Zarodki somatyczne			Number of somatic embryos per 1 explant Liczba zarodków na 1 eksplantat
		number liczba	%	length długość, mm	
Light Światło	MS0	12 bc B*	9.2	1.67 a A	0.40 bc B
	MS5	37 ac B	28.5	1.10 a A	1.23 ac B
	MS7	54 a A	41.5	1.88 a A	1.80 a A
	MS10	27 ac B	20.8	1.59 a A	0.90 ac B
Darkness Ciemność	MS0	3 bc B	9.1	1.70 a A	0.10 bc B
	MS5	8 ac B	24.2	1.20 a A	0.32 ac B
	MS7	11 a B	33.3	1.34 a A	0.44 a B
	MS10	11 a B	33.3	2.02 a A	0.44 a B

*means in columns marked with the same letter do not differ significantly at $\alpha = 0.05$, lower-case letter define the influence of 2,4-D under the same light conditions, upper-case letter define the influence of light between the same kinds of media

*średnie w kolumnach oznaczone tymi samymi literami alfabetu nie różnią się istotnie przy poziomie istotności $\alpha = 0,05$, małe litery dotyczą wpływu 2,4-D w obrębie tych samych warunków świetlnych, duże litery określają wpływ światła pomiędzy tymi samymi rodzajami pożywek

medium (MS0) and when exposed to light, as compared with the darkness. The number of roots and shoots, on the other hand, was higher on the control medium than on the media enriched with auxin.

Auxin 2,4-D significantly affected the number of regenerating embryos both when exposed to light and in the darkness (tab. 2). The effect of light conditions on the regeneration of somatic embryos was also found, but only on the MS7 medium. On the other media the light conditions affected neither the number of somatic embryos nor the number of embryos per explant. Most of them were produced in the light, on the MS7 medium, with an average of 1.8 embryos per explant. In the light, yellow-and-green embryos were dominant, while in the darkness – white or white-and-maroon.

The medium type did not affect the length of the embryos (tab. 2). Neither was there reported an effect of light conditions on that parameter. In the present experiment there were embryos produced at all the developmental stages (figs 1, 2, 3). Most of them were recorded at the globular stage (80%), followed by torpedo (10%), heart and cotyledonary stages (4% each) and at maturity (2%). It was shown that darkness delayed the formation and the maturity of embryos. In the light, on all the media with auxin they

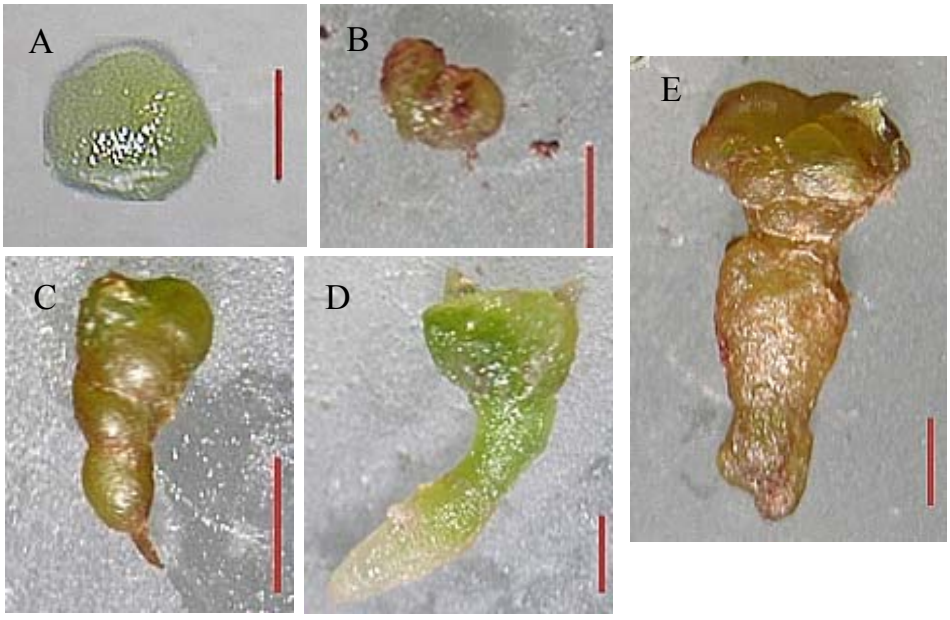

Fig. 1. Somatic embryos of *Astrophytum asterias* (Zucc.) Lem. obtained on the light: A – globular stage on the MS0 medium; B – heart stage on the MS5 medium; C – torpedo stage on the MS7 medium; D – cotyledonary stage on the MS0 medium; E – mature stage on the MS5 medium; 1 bar = 1 mm (magnification: A, B, C – 0.7 × 10, D – 1.2 × 10)

Ryc. 1. Zarodki somatyczne *Astrophytum asterias* (Zucc.) Lem., uzyskane na świetle: A – stadium globularne na pożywce MS0; B – stadium sercowate na pożywce MS5; C – stadium toredy na pożywce MS7; D – stadium laski na pożywce MS0; E – stadium dojrzałości na pożywce MS5; 1 bar = 1 mm (powiększenie: A, B, C – 0,7 × 10, D – 1,2 × 10)

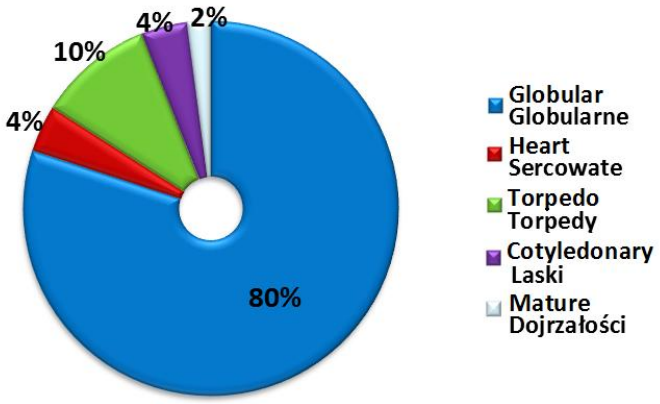

Fig. 2. Percentage share of different developmental stages of somatic embryos of *Astrophytum asterias* (Zucc.) Lem.

Ryc. 2. Udział procentowy poszczególnych stadiów rozwojowych of *Astrophytum asterias* (Zucc.) Lem. zarodków somatycznych u *Astrophytum asterias* (Zucc.) Lem.

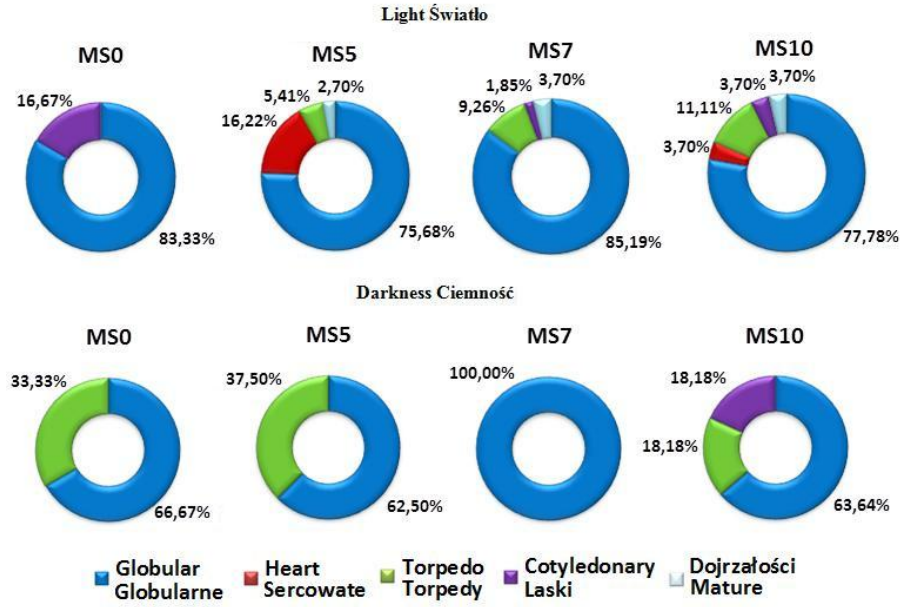

Fig. 3. Percentage share of developmental stages of somatic embryos of *Astrophytum asterias* (Zucc.) Lem. on different media

Ryc. 3. Udział procentowy stadiów rozwojowych zarodków somatycznych u *Astrophytum asterias* (Zucc.) Lem. na różnych pożywkach

reached maturity (up to 3.70%), however, only on the MS10 medium embryos occurred at all the developmental stages. The embryos incubated in the darkness did not reach such advanced stages. There was no presence of embryos at the heart stage and at maturity reported. None of the embryos isolated demonstrated a vascular connection with the explant they have regenerated from.

DISCUSSION

Over time, numerous cacti micropropagation techniques were applied, with various effectiveness: isolated meristems, lateral buds, adventitious shoots as well as somatic embryogenesis [Rubluo 1997, Giusti et al. 2002, Lema-Rumińska and Licznerska 2004]. Somatic embryogenesis, due to a considerable functional potential, offers a promising alternative [Jasrai et al. 2003]. Somatic embryogenesis was used in *Mediocactus coccineus* [Infante 1992], *Opuntia ficus-indica* [Linhares et al. 2005], *Ariocarpus kotschoubeyanus* [Moebius-Goldammer et al. 2003] as well as in *Copiapoa tenuissima forma monstruosa* [Lema-Rumińska 2011].

In general, it is assumed that darkness enhances the formation of somatic embryos [Linhares et al. 2005], while the conversion of embryos into plants requires transferring them into the light [Hoshino and Cuello 2005]. A positive effect of darkness on that process was demonstrated with the example of *Opuntia ficus-indica* [Linhares et al. 2005] and *Aloe vera* [Garro-Monge et al. 2008]. Our research in *A. asterias* does not coincide with those reports. The darkness led to a clear decrease in the number of regenerating embryoid structures and delayed their maturity. Under those conditions, obtaining embryos at the cotyledonary stage was successful only on the medium containing 10 $mg \cdot dm^{-3}$ 2,4-D. In the other cases globular stages dominated. Similarly in the light, however, embryos at the maturity stage were reported on all the media enriched with 2,4-D.

The investigations into the role of light in the initiation of somatic embryogenesis were performed by many authors [Linhares et al. 2005, Shi et al. 2009]. Light has a significant effect on the plant morphogenesis, also on the effectiveness of *in vitro* cultures, which was shown in the present study with *A. asterias*. Our use of the intensity of quantum irradiation of 24.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$for fluorescent lamps emitting daylight demonstrated a positive effect on the induction of embryogenesis. Positive results were also reported in *Aztekium ritteri* [Santacruz-Ruvalcaba et al. 1998] as well as in *Turbinicarpus pseudomacrochele* [Torres-Munoz and Rodriguez-Garay 1996]. In other species both the inducing and the suppressor effects of light conditions were observed. It also happens that the light does not have a significant effect on the efficiency of embryogenesis at all, which, however, was not confirmed for the cactus investigated. In *Cinnamomum camphora* [Shi et al. 2009], the light conditions did not affect the total efficiency of somatic embryogenesis, however, in the light more embryos regenerated from a single explant. In the present research we observed an enhancement of both parameters. In the light on the MS7 medium we obtained an average of 1.80 embryos per explant, while, in the darkness only 0.44. A similarly suppressor nature of the darkness was confirmed by D'Onofrio et al. [1998 cited after Hoshino and Cuello, 2005] follow-

ing the example of *Sidonia* sp., who reported 0.1 embryo from a single explant in the darkness and 0.4 – in the light. In the present research the light also enhanced the number of explants forming embryoid structures; it accounted for 40.0% and 26.7%, respectively. Similarly in the experiment reported by Lema-Rumińska and Fijałkowska [2006] which involved *Gymnocalycium mihanovichii,* the embryos were more frequently produced in the daylight, with 62.5% of the explants, than in the darkness (only with 29.2%).

Stress is yet another essential factor affecting the efficiency of somatic embryogenesis [George et al. 2008]. The injury which comes with cutting the explant into half (or even during its isolation), which was reported in the present study, is the first signal received by the cells, connected with acquiring the competencies for embryogenesis [Pua and Davey 2010], which was demonstrated in *Opuntia* by Linhares et al. [2005]. Undamaged explants produced, depending on the concentration of Picloram, from 0.0 to 1.33 embryos, with the maximum of 41.67% of explants. However, after the mechanical damage of the tissue explants regenerated from 0.06 to 5.22 embryos. There was also an increase, up to 83.33%, in the share of explants forming embryoid structures recorded. The treatment was also successful in the present research. In *A. asterias* the embryoid structures regenerated only on the surface of the explant cutting. Furthermore, in our research, somatic embryos regenerated also on the MS0 medium, without growth regulators. This fact could have been due to the effect of the mechanical damage of the explant tissues. Santarem et al. [1997], on the other hand, claim that injuring the tissue does not only increase the efficiency of embryogenesis, but mostly accelerates the process of the formation of embryos, which could coincide with the present research; the first embryoid structures appeared already in the 3rd week of the observations, on the MS7 medium. As for *Turbinicarpus pseudomacrochele*, somatic embryos were produced after the 4th week of the culture [Torres-Munoz and Rodriguez-Garay 1996]. In other species they regenerated only after a few months, e.g. in *Mammillaria sanangelensis*, after 90 days [Marin-Hernandez et al. 1998].

The *in vitro* germinated seedlings applied at the second stage of the present research are frequently used as explants in cacti tissue cultures. According to Rubluo [1997], they are a perfect material for a further embryo regeneration, which was confirmed in the present research. Thanks to the presence of the meristematic tissue, they ensure a greater genetic stability and are a better source of sterile explants [Rubluo 1997]. Besides the species investigated, the explants derived from their fragments turned out to be applicable in somatic embryogenesis in *Mediocactus coccineus* [Infante 1992] and in *Agave victoriae-reginae* [Martinez-Palacios et al. 2003]. In *Catharanthus roseus* it was even found that the seedlings germinated *in vitro* are the only explant which forms an embryogenic callus [Aslam et al. 2009].

Garro-Monge et al. [2008] demonstrate that the induction of the process mostly depends on the auxins. One shall stress, however, that respective auxins vary in their capacity for it [Sage et al. 2000]. Auxin 2,4-D is considered to be most effective in the initiation of the process [Jasrai et al. 2003]. The results of the present research also confirm those assumptions. On the media enriched with auxin there were, on average, three-times more embryos produced than on the control medium. However, their effect also depends on the species; for example Lema-Rumińska [2011] and Lema-Rumińska

and Fijałkowska [2006] report on the regeneration of embryoid structures from the meristematic explants in *Copiapoa tenuissima* Ritt. *f. monstrosa* and *Gymnocalycium mihanovichii f. aurantica*, after applying the MS medium containing 2,4-D at the concentration of 2.0 mg·dm^{-3}. As for the meristematic explants in *Cereus peruvianus,* the application of a medium with 4.0 mg·dm^{-3} 2,4-D only helped the formation of non-embryogenic callus and sporadic adventitious shoots [Karimi et al. 2010]. Our research suggests that at least in the case of that regulator, determining the optimal concentration for the induction of somatic embryogenesis in *A. asterias* was successful since above 7 mg·dm^{-3} the number of embryos (depending on the light conditions) did not change.

CONCLUSIONS

1. The process of somatic embryogenesis in *Astrophytum asterias* (Zucc.) Lem. depends on light conditions and the content of exogenous auxin 2,4-D.
2. The number of explants regenerating somatic embryos was greater on the media with auxin 2,4-D added than on the control medium without growth regulators and when exposed to light rather than to darkness.
3. The number of explants regenerating shoots and roots was greater on the control medium without growth regulators than on the medium with auxin 2,4-D added.
4. The most number of embryos per 1 explant (1.8) were obtained on the MS7 medium (7 mg·dm^{-3} 2,4-D) in the light conditions.

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INDUKCJA EMBRIOGENEZY SOMATYCZNEJ U *Astrophytum asterias* (Zucc.) Lem. W ASPEKCIE WPŁYWU WARUNKÓW ŚWIETLNYCH ORAZ STĘŻENIA AUKSYNY 2,4-D

Streszczenie. *Astrophytum asterias* (Zucc.) Lem. jest jednym z najbardziej pożądanych przez producentów i kolekcjonerów kaktusów na świecie, a jednocześnie gatunkiem zagrożonym wyginięciem w środowisku naturalnym. Techniki mikrorozmnażania mogą być pomocne zarówno w jego ochronie *ex situ*, jak i przyczynić się do jego rozpowszechnienia na rynku, zaspokajając potrzeby hodowców i kolekcjonerów kaktusów. Embriogeneza somatyczna jest najefektywniejszą spośród metod mikrorozmnażania i polega na tworzeniu zarodków somatycznych z komórek wegetatywnych. Pożywka, warunki świetlne oraz rodzaj eksplantatu, mają kluczowy wpływ na jej wydajność. W indukcji embriogenezy najczęściej stosowaną jest auksyna 2,4-D (kwas 2,4-dichlorofenoksyoctowy). W badaniach określono wpływ jej stężenia i warunków świetlnych na wydajność embriogenezy somatycznej. Nasiona wykładano na zmodyfikowaną pożywkę MS o zredukowanej zawartości makroelementów oraz sacharozy ½MS (pH 5,7 – przed autoklawowaniem). Wszystkie kultury *in vitro* inkubowano w pokoju wzrostowym (24 ± 2°C, 16 h dzień/ 8 h noc fotoperiod, natężenie napromienienia kwantowego: 24,3 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Po 14 dniach z 70% nasion uzyskano siewki. W celu regeneracji zarodków somatycznych, połówki zielonych siewek wyłożono na zmodyfikowaną pożywkę MS z dodatkiem auksyny 2,4-D w stężeniu: 5; 7 i 10 $mg \cdot dm^{-3}$, kontrolę stanowiła pożywka MS0 bez regulatorów wzrostu. W celu zweryfikowania wpływu warunków świetlnych, połowa eksplantatów była inkubowana na świetle, a reszta w ciemności. Po 10 tygodniach trwania kultury wyizolowano, zliczono i zmierzono zregenerowane zarodki. Uzyskano je na wszystkich pożywkach w obu warunkach świetlnych. Badania własne potwierdziły pozytywny wpływ 2,4-D i światła na liczbę eksplantatów tworzących struktury embrioidalne oraz na liczbę regenerujących zarodków. Najwięcej zarodków na jednym eksplantacie (1,8) otrzymano na pożywce MS7 w warunkach światła.

Słowa kluczowe: *Astrophytum asterias*, zarodki somatyczne, kaktus, mikrorozmnażanie

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