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Plant growth regulator (PGR) producing alga strains as alternative hormone sources for application in tissue cultures of higher plants

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1. INTRODUCTION

The arable land per capita decreased by about 44% in the past 40 years as a consequence of a dynamic increase in the world's population and the limited surface on the Earth that can be used for agriculture. The ever increasing demand for food should be met with higher plant production, which can be realised with environment-friendly and intensive cultivation technologies of high-yielding genotypes that comply with all quality and food safety parameters. To achieve this goal, an important condition for genetic improvement is the generation of homozygous lines that may be useful parental material for improved cultivars and hybrids via the combination of traditional and advanced technologies such as the haploid induction methods anther and microspore culture, and distant (intergeneric) hybridisations. Via the use of these technologies, plant breeders could adapt more easily to the changes of the market and demands of the producers because breeding time becomes shorter and the production cost of new hybrids and cultivars is significantly lower. The haploid breeding methods have become efficient tools to generate homozygous dihaploids (DH) in a number of important crop plants. At present, 207 DH cultivars are cultivated worldwide. The higher the efficiency of the haploid breeding techniques the higher the number of homozygous lines produced. For this purpose, natural (organic) supplements to culture media have long been applied due to their hormone-like effects. These substances are not essential for tissue growth but influence the timing and intensity of plant growth and development, and thus alone or in combination with other ingredients of the culture medium increase the efficiency of *in vitro* plant production. However, natural supplements to tissue cultures must have a constant and

reproducible composition, which can be achieved by growing the source organism under controlled conditions. The artificial production and selection of edaphic alga strains on the basis of bioassays for hormone-like effects is a prerequisite for the application of their biomass as natural supplements in plant tissue cultures.

2. OBJECTIVES

The aim of the experimental work underlying this dissertation was to develop the application of the biomass from hormone-producing microalga and cyanobacterium strains in anther culture media in order to increase the efficiency of producing plant breeding material.

Therefore, we set out the following objectives:

1 screening of the Mosonmagyaróvár Alga Culture Collection (MACC) for microalga and cyanobacterium astrains with auxin- and cytokinin-like effects by using bioassays,

2 quantitative as well as quantitative and qualitative determination of indole-3-acetic acid (IAA) and cytokinins, respectively, by analytical methods in MACC strains exerting the highest hormon-like effects,

3 investigation of the potential application of biomasses from MACC strains with proven hormone content and hormonal effect in culture media of maize and wheat anther cultures, for the purpose of increasing haploid induction and plant regeneration, and thereby improving the efficiency of plant breeding material production.

3. MATERIALS AND METHODS

3.1. Hormone analysis of microalga and cyanobacterium strains

Demonstration of auxin- and cytokinin-like effects by bioassays

For the demonstration of auxin- and cytokinin-like effects in 252 MACC strains, the excised cucumber cotyledon root formation bioassay (Zhao et al., 1992) and the excised cotyledon expansion bioassay of cucumber and radish (Letham, 1971; Zhao et al., 1992) were used, respectively.

Determination of cytokinins and IAA by analytical methods

Quantitative and qualitative determination of cytokinins was performed by high pressure liquid chromatography-mass spectrometry (HPLC-MS), the quantitative determination of IAA was done with gas chromatography-mass spectrometry (GC-MS) (Bartók et al., 1996).

3.2. *In vitro* anther cultures

The MACC microalga és cyanobacterium strains selected:

Microalgae:	553 (*Klebsormidium flaccidum*)
	560 (*Chlorella* sp.)
	583 (*Neochloris* sp.*)*
Cyanobacteria:	642 (*Leptolyngbya* sp.*)*
	643 (*Anabaena* sp.)

Plant material:

Maize:	H1: DH 240 × DH 314
	H2: DH 109 × DH 314
	H3: DH 105 × HMv 5405
Wheat:	Benoist
	Mv Pálma

Culture media:

Maize induction media:

- **A:** YP medium (Ku et al., 1981) as modified by Genovesi and Collins (1982) and supplemented with 0.1 $mg \cdot l^{-1}$ of 2,3,5-triiodobenzoic acid (TIBA),
- **B_1:** YP medium as modified by Dieu and Beckert (1987) and supplemented with 0.1 $mg \cdot l^{-1}$ of 2,3,5-TIBA, or
- **B_2:** as B_1 but supplemented with 2 $mg \cdot l^{-1}$ of 2,4-dichlorophenoxy acetic acid (2,4-D), or
- **B_3:** as B_1 but supplemented with 1 $mg \cdot l^{-1}$ of kinetin (KIN) and 0.5 $mg \cdot l^{-1}$ of 1-naphthalene acetic acid (NAA)

Wheat induction media:

- W_{14} medium containing 2 $mg \cdot l^{-1}$ of 2,4-D and 0.5 $mg \cdot l^{-1}$ of KIN (Ouyang et al. 1989)

Maize regeneration:

- modified N6 medium (Chu, 1978) supplemented with 1 $mg \cdot l^{-1}$ of KIN and 0.5 $mg \cdot l^{-1}$ of NAA

Wheat regeneration:

- 190-2 medium containing 0.5 $mg \cdot l^{-1}$ of KIN and 0.5 $mg \cdot l^{-1}$ of NAA (He and Ouyang, 1983)

Control media were used as above whereas treatments with algal strains contained either 50% hormone concentration and 1 $g \cdot l^{-1}$ dry weight of algal biomass or 2 $g \cdot l^{-1}$ dry weight of algal biomass without hormone supplement.

3.3. Morphological and cytological analysis of structures induced from maize microspores

The embedding of stuctures was done according to standard techniques (Spurr, 1969).

Ploidy levels were determined by a FACScan flow cytometer (Beckton-Dickinson). The results were analysed by the CellQuest software (Beckton-Dickinson).

3.4. Statistical analysis

The data were statistically evaluated with the SPSS for Windows software (version 10.0).

4. SUMMARY OF RESULTS AND CONCLUSIONS

4.1. The IAA content of microalga and cyanobacterium strains

The auxin-like effect of soil-borne microalgae and cyanobacteria has up to now been studied mainly indirectly, i.e. by colorimetry to indicate the presence of indolyl compounds and by bioassays to demonstrate auxin-like effects (Misra and Kaushik, 1989; Stirk et al., 2002). There is only one paper on direct IAA detection in a free-living diazotrophic cyanobacterium

strain by GC-MS (Sergeeva et al., 2002).

The IAA content in the microalgae and cyanobacteria selected in this work on the basis of bioassays for auxin-like activity was higher than in the *Nostoc*269 strain studied by Sergeeva et al. (2002). The MACC 355 strain, which produced the highest auxin amount, contained 23 times more IAA than the above strain. To our knowledge, this is the **first demonstration of IAA production in a soil-borne *Leptolyngbya* species**. With the exception of the MACC 355 strain, **a strong correlation was found between indole-3-butyric acid equivalents in the bioassays and the IAA content measured by GC-MS, which supports the reliability of the excised cucumber cotyledon root formation bioassay for demonstrating auxin-like activities**. The deviation in the case of the MACC 355 strain could be explained by the suppressing effect of supra-optimal IAA concentrations.

4.2. Quantitative and qualitative determination of cytokinin contents in microalga and cyanobacterium strains

Cytokinin composition of plants has changed during evolution (Auer, 1997). Based on their cytokinin content plants can be divided in two main groups. Microalgae, ferns and lichens contain free bases of isopentenyl-adenin and zeatin or their ribosides, whereas higher plants (gymnosperms and angiosperms) are composed of more complex cytokinins: isopentenyl-adenine, zeatin, dihydrozeatin and their derivatives, such as ribosides, O- and N-glycosides (Auer, 1997). According to Mok és Mok (2001) the synthesis of benzyladenine and benzyladenine riboside is also the characteristics of higher plants.

Our results in part confirm the above statements, but also contradict to some of them. The more studies are performed on the cytokinin content

of microalgae and cyanobacteria, the more likely is the rejection of the cytokinin evolution theory, though definite conclusions can be drawn only after a large number of studies.

Microalgae and cyanobacteria studied in this thesis did not contain isopentenyl-adenine (iP). Only two *Chlorella* strains contained isopentenyl-adenosine (iPR). This confirms the observation of Stirk et al. (2002) that their set of microalgae and cyanobacteria contained very little iP and iPR, but contradicts to the results of Auer (1997) and Ördög et al. (2004) who found iP and its riboside in all microalgae studied.

Among free zeatins, as revealed by our measurements and in agreement with previous data, the *cis* isomeric form dominated (by about 2.5 to 12 times higher amount) over the *trans* isomer. A different trend was characteristic for the zeatin ribosides, among which the two isomers were present in the samples at nearly identical proportions.

Though all strains contained 2-hydroxy-zeatin, it was the only cytokinin with quantitative difference between strains belonging to *Chlorophyta* and *Cyanobacteria.* In our opinion, the reason for this difference lies in the eukaryotic origin of microalgae, but its confirmation needs further elucidation.

Of the eight strains studied, we found dihydrozeatin (DZ) in five and dihydrozeatin riboside (DZR) in seven of them. **For the first time, we demonstrated the presence of DZ in *Anabaena* sp. and DZR in *Leptolyngbya* cyanobacterium strains.** This is in contradiction with previous findings because the presence of DZ has been attributed specifically to higher plants (Auer, 1997), although Ördög et al. (2004) also detected it in a microalga strain.

Benzyladenine production in soil-borne microalgae was first reported by Ördög et al. (2004). Our results confirm this observation as we detected BA in four out of the six microalga strains studied. **We found BA and benzyladenine riboside for the first time in a cyanobacterium**, and this in the MACC 643 *Anabaena* strain, which showed the strongest cytokinin-like activity as well as the highest total cytokinin content.

Based on their mass spectra, we detected three novel isopentenyl type cytokinins in microalga and cyanobacterium strains. Their identification via chemical synthesis is in progress. They probably possess high cytokinin activity, which may explain the high cytokinin-like effect in the MACC 643 strain. This activity will be verified by bioassays following chemical synthesis.

The cytokinin content measured in the MACC 642 strain with high cytokinin-like activity was very low. The reason for this high activity could be, in our opinion, an undetected or a yet unknown cytokinin compound or some other factors.

We were interested to find out whether the microalga strains with different cytokinin-like activity also differed in their cytokinin composition. For this purpose, besides strains selected by bioassays for high cytokinin-like activity we also analysed by HPLC-MS MACC strains with auxin-like activity and auxin content but no cytokinin-like activity in bioassays. There was no quantitative or compositional difference in cytokinin content between strains possessing cytokinin-like activity and those without this activity, which confirmed the results of Ördög et al. (2004). As the interaction between auxins and cytokinins ranges from synergy to antagony (Nordström, 2004), IAA might have played a suppressive role in the cytokinin-like activity. Also, it can not be excluded that the strains studied

synthesised other inhibitory compounds that influenced the uptake and activity of cytokinins.

4.3. The applicability of MACC strains in cereal anther cultures

When comparing induction media in maize anther cultures, we observed that the auxin transport inhibitor 2,3,5-TIBA decreased the number of microspore-derived structures and regenerated plants relative to those induced by the synthetic auxin 2,4-D. This observation is in agreement with the results of Bouharmont (1977) and Choi et al. (2001) who reported the inhibitory effect of 2,3,5-TIBA on embryo development. The *in vitro* response (anther induction and frequency of microspore-derived structures in relation to total inoculated anthers) of B_3 medium supplemented with KIN és NAA synthetic hormones was inferior compared to the effect of the A, B_1 and B_2 culture media containing 2,3,5-TIBA or 2,4-D. Thus, the amount of hormones in the B_3 medium was not sufficient to induce a strong anther response.

Though the anther response of three maize hybrids (H1, H2, H3) showed some specific dependence on the MACC strains, the 1 mg·l^{-1} of 2,4-D treatment supplemented with 1 g·l^{-1} of MACC 643 strain resulted in a universally and significantly increased anther response in all three hybrids. The MACC 643 strain alone did not exceed the effect of the combination with 2,4,-D in any of the experiments. We concluded that the significantly improved effect over the control was caused by the synergistic effect between 2,4-D and hormones or hormone-like compounds in the MACC 643 cyanobacterium. In the case of the low induction capacity H3 hybrid the anther response was higher with all MACC strains when

combined with 2,4-D. The reason might lie in the high endogenous auxin content of this low responsive genotype, which can be suddenly elevated only by the addition of 2,4-D.

In the two wheat cultivars studied, similarly to the H3 maize genotype, the combined 2,4-D plus microalga or cyanobacterium treatments were more effective than the hormone-free treatments containing only microalgal or cyanobacterial biomass. **Examining the effect of MACC strains on the haploid induction capacity of wheat**, again **the 1 mg·l^{-1} of 2,4-D treatment supplemented with 1 g·l^{-1} of MACC 643 strain was significantly better than the control.** In our opinion, the synergistic effect between the hormones in the algae and the 2,4-D was responsible for the increased *in vitro* anther response in wheat as well.

Although the studied genotypes showed positive response to the same alga strain(s) independently of the growing conditions of the donor plants, it seems reasonable to study the effect of bioactive substances in anther culture with donor plants raised under controlled conditions. Plant material grown outdoors may give results that are difficult to evaluate statistically (low reproducibility, seasonal effects, etc.). These biotic and abiotic environmental factors may affect the anther response and mask the supplementary effect of the natural culture medium to be studied.

4.4. The identification of microspore-derived structures which result in the maximum number of spontaneous dihaploid maize plants

In maize anther culture experiments we observed the development of structures with various morphology from microspores following the sporophytic pathway. These structures could be grouped in the following

four morphotypes: white and translucent, white and compact, yellow and translucent, yellow and compact. Our analysis was directed to answer two questions: 1. is there a relation between the morphological characteristics and ploidy of these structures and their plant regeneration capacity, 2. in which phase of *in vitro* culture do ploidy changes of microspores or microspore-derived structures occur?

Our results, in accordance with those of Brisibe et al. (2000) obtained with wheat embryogenic callus lines, demonstrated that neither the growing conditions of donor plants, nor the different induction media had a major influence on the statistical distribution and regeneration capacity of the different microspore-derived morphotypes, which indicates that these traits are genetically determined. **The white and compact morphotype, comprising one third of the induced structures, regenerated plants at a frequency of 52%, and 43% of these plants proved dihaploid.** Both the average plant regeneration frequency and the proportion of dihaploid plants in the other morphotypes was significantly lower.

We determined that the changes in chromosome numbers and genome duplications had already happened by the third week of culture after induction.

By analysing embedded tissue sections, we observed dividing microspores that merged with other induced structures. This confirms the observation of Wilson et al. (1978) that the main reason for mixoploidy in microspore-derived structures could be the contribution of more than one microspore to these structures.

5. ORIGINAL SCIENTIFIC RESULTS

1. Microalga and cyanobacterium strains producing indole-3-acetic acid were selected from the Mosonmagyaróvár Alga Culture Collection (MACC).

   For the first time, the presence indole-3-acetic acid was demonstrated in *Leptolyngbya* cyanobacterium.

2. Microalga and cyanobacterium strains producing cytokinins were selected via bioassays from the MACC.

   For the first time, the presence of aromatic cytokinins, benzyladenine and benzyladenine riboside, was demonstrated in *Anabaena* cyanobacterium.

   We demonstrated for the first time the presence of dihydrozeatin in *Anabaena* cyanobacterium, and dihydrozeatin as well as dihydrozeatin riboside in *Leptolyngbya* cyanobacterium.

   Based on their mass spectrum, novel isopentenyl type cytokinins were identified in microalga and cyanobacterium strains.

3. The synthetic auxin 2,4-D could be substituted in maize anther cultures by the biomass of MACC 560, 642 and 643 microalga and cyanobacterium strains. Optimal combinations of strain 643 and 2,4-D significantly increased haploid induction rates.

In wheat anther cultures, a combined treatment of 1 $g \cdot l^{-1}$ of strain 643 + 1 $mg \cdot l^{-1}$ of 2,4-D decreased the amount of 2,4-D synthetic auxin required for the induction of an *in vitro* response.

4. We determined that 2-3 mm white and compact morphotypes of microspore-derived structures produced the highest number of spontaneous dihaploid regenerated maize plantlets, which are desirable for practical applications.

   We determined that genome duplication in induced maize microspore takes place by the third week of *in vitro* culture.

6. POTENTIAL APPLICATIONS

***Via* the 1 $g \cdot l^{-1}$ of MACC 643 + 1 $mg \cdot l^{-1}$ of 2,4-D treatment, a high number of homozygous lines can be produced** to generate new hybrids and cultivars by increasing the anther response in maize and wheat genotypes of high agronomical value but with low haploid induction capacity.

By the selection of microspore-derived **structures on the basis of morphotypes the work input and operational cost can be decreased in maize anther culture.**

We are convinced that the a **combined application of microalgal and cyanobacterial extracts** together with the selection of microspore-derived structures significantly **increase the efficiency of anther culture**.

The IAA- and cytokinin producing **microalga and cyanobacterium strains** selected in our experimental work for promoting haploid induction and embryogenesis in cereals **will be studied in plant regeneration from wheat zygotes produced by *in vitro* gamete fusion** or after **microinjection**.

It is important to study the identified and in cereal anther culture positive **MACC strains in** the *in vitro* culture of **other plant species**. In the first place, their application is suggested in the micropropagation of ornamental plants and fruit stocks because an increased efficiency in these cases could results in **economical savings**. If the positive effects are proven on intact plants as well, these strains may indeed be **utilised in agriculture**.

7. LIST OF PAPERS PUBLISHED IN THE TOPIC

REVIEWED PUBLICATIONS IN SCIENTIFIC JOURNALS

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2. **Jäger, K**., Bartók, T., Ördög, V., Barnabás, B. Improvement of maize *(Zea mays* L.) anther culture responses by algae-derived natural substances. Plant Sci. (under review)
3. Bakos, F., **Jäger, K.,** Barnabás, B. Regeneration of haploid plants from activated egg cells of wheat via zygote rescue. Acta Biol. Cracov. (accepted)

4. **Jäger, K**., Ördög, V. Barnabás, B. Effect of algae-derived natural substances on anther culture response of wheat (*Triticum aestivum* L.). Acta Agron. Hung. (accepted)
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CONFERENCE PUBLICATIONS

PRESENTATION

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3 Lepossa, A. and **Jäger, K**. 2000. New potential applications of microalgae in agriculture. European Science Foundation Summer School and Workshop, 27 August-3 September 2000, Ballywaughan, Co. Clare, Ireland. Programme and Abstracts p. 52.

4 **Jäger, K**. and Ördög, V. 2001. Seaweed products and terrestrial algae — comparison of cytokinin-like activities. XVIIth International Seaweed Symposium, 28 January-2 February 2001, Cape Town, South Africa.

5 Molnár, Z., **Jäger, K**., Barnabás, B., Ördög, V. 2001. Growth promoting effects of microalgal and cyanobacterial suspensions on tissue cultures of pea and maize. 4th International Symposium in the Series Recent Advantages in Plant Biotechnology, September 17-21 2001, Třeboň, Czech Republic. Book of Abstracts p. 129.

6 **Jäger K**., Ördög V., Barnabás B. 2003. Improvement of anther culture responses by algae derived natural substances. European Society of Microalgal Biotechnology, 5th European Workshop, Biotechnology of Microalgae, June 23-24 2003, Bergholz-Rehbrücke, Germany. Abstracts.

7 **Jäger K**., Ördög V., Barnabás B. 2003. Improvement of anther culture responses by algae derived natural substances. XIth International Conference on Plant Embryology "Plant Reproduction: From Mendel

to Molecular Biology" September 1-3 2003, Brno, Czech Republic. Book of Abstracts 116a.