DOI: 10.12905/0380.phyton61-2022-109 Published online 15 Mar 2022

Does biostimulant Agrostemin® exhibit plant growth regulator activities?

Jana Ambrožič-Dolinšek[1,2,3], Domen Ornik[1], Rebeka Branda[1], Zoltán Molnár[4] & Terezija Ciringer[1]

with 3 figures

Key words: Bioassay, Agrostemin, biostimulants, plant growth regulators, auxin, cytokinin.

Summary

Ambrožič-Dolinšek J., Ornik D., Branda R., Molnár Z. & Ciringer T. 2022. Does biostimulant Agrostemin® exhibit plant growth regulator activities? – Phyton (Horn, Austria) 61: 109–116, with 3 figures.*

Agrostemin® is a natural plant preparation used as a plant biostimulant (PBS) in agriculture. It elicits many positive responses, including yield increases, and is proposed to optimize metabolic processes, growth and development, and stress resistance. The aim of the present study is to identify and quantify whether it exhibits auxin-like and cytokinin-like plant growth regulator (PGR) activities. The 'Excised Cucumber Cotyledon Root Formation Bioassay' and the 'Mung Bean Root Formation Bioassay' were used to determine the auxin-like activities. The water extract of Agrostemin® showed auxin-like activity in both bioassays. The activity in the cucumber bioassay was equivalent to about 0.3 mg L^{-1} IBA, and in the mung bean bioassay it was equivalent to about 0.5 to 1 mg L^{-1} IBA. The 'Excised Cucumber Cotyledon Expansion Bioassay' and the 'Triticum Leaf Chlorophyll Retention Test' were used to determine the cytokinin-like activities of Agrostemin®, but both showed no cytokinin-like activity.

1. Introduction

Agricultural growing practices have evolved towards organic, sustainable, or other environment-friendly systems. Natural plant biostimulants, based on natural substances, have been proposed as an innovative solution for sustainable agriculture and have received considerable attention from both the scientific community and commercial companies, especially in the last two and a half decades but also earlier (Yakhin & al. 2016).

One of the most innovative and promising solutions to address these important challenges is the use of plant biostimulants (PBS), which are described as "materials which contain substance(s) and/or microorganisms, whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and/or crop quality, independent of its nutrient content" (European Biostimulant Industry Council 2016). The formulations of plant biostimulants are generally proprietary compositions based on algal extracts, complex organic materials, plant hormone-like compounds, amino acids, and humic acids (Povero & al. 2016). Biostimulants can contain traces of natural plant hormones, but their biological action should not be ascribed to them, otherwise they would have to be registered as plant growth regulators (PGRs) (Bulgari & al. 2015).

The composition of several biostimulants is partly unknown. The complexity of the extracts and the large number of molecules contained in the solution make it very difficult to understand the most active compound. Therefore, the classification of biostimulants should be based on their action in plants or, even better, on the physiological responses of plants rather than on their composition (Bulgari & al. 2015). This is especially true for the older preparations from the 1980s of the last century, such as Agrostemin® (Patent Document No. 32749, 1974). The producers describe Agrostemin® as a broad-spectrum bioenergent, a promising natural bioregulator, a natural fertilizer, or as a bioactivator, a positive allelopathic substance, and a plant preparation used to improve plant productivity (Agrostemin 2001). Today these types of substances are called

[1]) University of Maribor, Faculty of Natural Sciences and Mathematics, Koroška cesta 160, 2000 Maribor, Slovenia; corresponding author: J. Ambrožič-Dolinšek (e-mail: jana.ambrozic@um.si)

[2]) University of Maribor, Faculty of Agriculture and Life Sciences, Pivola 10, 2311 Hoče, Slovenia

[3]) University of Maribor, Faculty of Education, Koroška 160, 2000 Maribor, Slovenia

[4]) Széchenyi István University, Faculty of Agricultural and Food Sciences, Vár Sqr. 2, 9200 Mosonmagyaróvár, Hungary

*) Printed volume published 15 Mar 2022

with common name biostimulants (Yakhin & al. 2016).

Agrostemin® is used worldwide as a biostimulant for several agricultural crops. It is produced under the Patent Law No. 32749 of the Yugoslav Federal Patent Office under the ownership of the inventor of Agrostemin®, Danica Gajić (Patent Document No. 32749, 1974) on the ground of the former SFRJ (Socialist Federal Republic of Yugoslavia), therefore it may not be valid any longer. The producer promotes it as no fertilizer, pesticide and/or hormone. According to a chemical analysis, Agrostemin® consists of two groups of organic compounds, the active complex and the inhibitors. The active complex is a mixture of free amino acids and derivatives of amino acids, and organic acids and derivatives of organic acids. The inhibitors in traces are derivatives of ABA (abscisic acid), saturated aliphatic hydrocarbons and cyclic inhibitor ($C_8H_{29}N_3O_7$) in trace amounts (Agrostemin 2001, 'General Data' and 'PRIMER – Review of application and achieved effects'). The formulation of the product 'profi' is a mixture of 4 % of the active complex and 96 % of the neutral carrier substance Mg_2SiO_4 (Agrostemin 2001, 'Structure').

The biological influences of Agrostemin® are expressed through the effects of its application on plants, as the increase of yield and quality of arable crop, fruit yield (number and size of fruit) and yield of industrial crop, as well as better management of meadow and pasture. It is proposed that it optimizes basic metabolism processes (respiration, assimilation, photosynthesis etc.), autotrophic and heterotrophic stages of development, and mineral nutrition, leading to better growth and development (Agrostemin 2001). It has positive effects on seed germination and on the treated soil, by transformation of nutrients into more accessible forms. Treated plants also gave higher yields and showed better resistance to disease, frost, drought, flooding and wounding (Mangotić 2003). In 30 years of practice, it has no negative effects on people, crops and the ecosystem (Mangotić 2003).

Agrostemin® is a biostimulant that may also contain trace amounts of natural plant hormone-like substances. To find out whether they have this kind of active role in plants, we would like to find out whether their application should elicit a specific physiological response characteristic of a particular group of plant growth regulators (Stirk & al. 2014). Therefore, the purpose of the present study was to examine whether the Agrostemin® preparation has plant growth regulators, cytokinin- or/and auxin-like activity, and to what extent its activity is comparable to synthetic plant growth regulators (PGRs) commonly used in plant biotechnology.

2. Materials and methods

Agrostemin preparation. The manufacturer's recommended dose (Agrostemin 2001) is 0.5 g of Agrostemin® in 1 L of water (500 mg L^{-1}). We tested another higher concentration and three dilutions of Agrostemin® in deionized water: 5000, 500, 50, 5 and 0.5 mg L^{-1}. Deionized water was used as control.

Biological auxin-like activity. For auxin-like activity of Agrostemin® the 'Excised cucumber cotyledon root formation bioassay' (Zhao & al. 1992) and the 'Mung bean root formation bioassay' (Hess 1961) were used.

The Excised Cucumber Cotyledon Root Formation Bioassay (Zhao & al. 1992): Cucumber (*Cucumis sativus* L.) seeds, Semenarna Ljubljana (www.semenarna.com), 'Kumare Dolge zelene', with an expected germination time of 10–14 days, were germinated in the dark. The seeds were washed with cold tap water for one hour. Then they were placed on 5 layers of paper towels, moistened with deionized water, on the bottom of plastic laboratory trays in the dark for germination. Cotyledons excised from 10-day-old seedlings were placed in a 6 cm Petri dish on a filter paper disk and treated with 3 mL auxin, indole-butyric acid (IBA) (0, 0.1, 0.3, 0.5, 1 and 3 mg L^{-1}), or with various dilutions of Agrostemin® (0, 0.5, 5, 50, 500, 5000 mg L^{-1}) dissolved in deionized water. Cotyledons were incubated in the dark at 25 ± 2 °C for 7 days to determine the auxin-like activity. Deionized water was used as control. The number of roots was counted and compared with the activity of the IBA control. Each treatment was replicated four times and the experiment was repeated three times.

The Mung Bean Root Formation Bioassay (Hess 1961): Mung bean (*Vigna radiata* (L.) R. Wilczek) seeds were washed with cold tap water for 3 hours, soaked in water for 6 hours and sown into the vermiculite. The seeds were then planted 1 cm deep in moistened vermiculite in plastic trays and placed at 25 °C in the light/dark period (16/8 h) with a light intensity of 30–60 µmol m^{-2} s^{-1} for about 7–10 days. The seeds were watered regularly. For the bioassay, uniform seedling cuttings with carefully removed cotyledons were used with 6–7 cm long hypocotyls cut 3 cm below the cotyledons. Four seedling cuttings were immersed for 24 h in 4.5 ml (25 mm × 90 mm) glass vials containing 10 ml indole butyric acid (IBA) (0, 0.1, 0.3, 0.5, 1, 3, 5 and 10 mg L^{-1}) or different dilutions of Agrostemin® (0, 0.5, 5, 50, 500, 5000 mg L^{-1}) dissolved in deionized water. Deionized water was used as control. The vials are returned to the original growth conditions for 24 hours. The seedlings are then rinsed with distilled water and returned to the same vials with 10 ml dis-

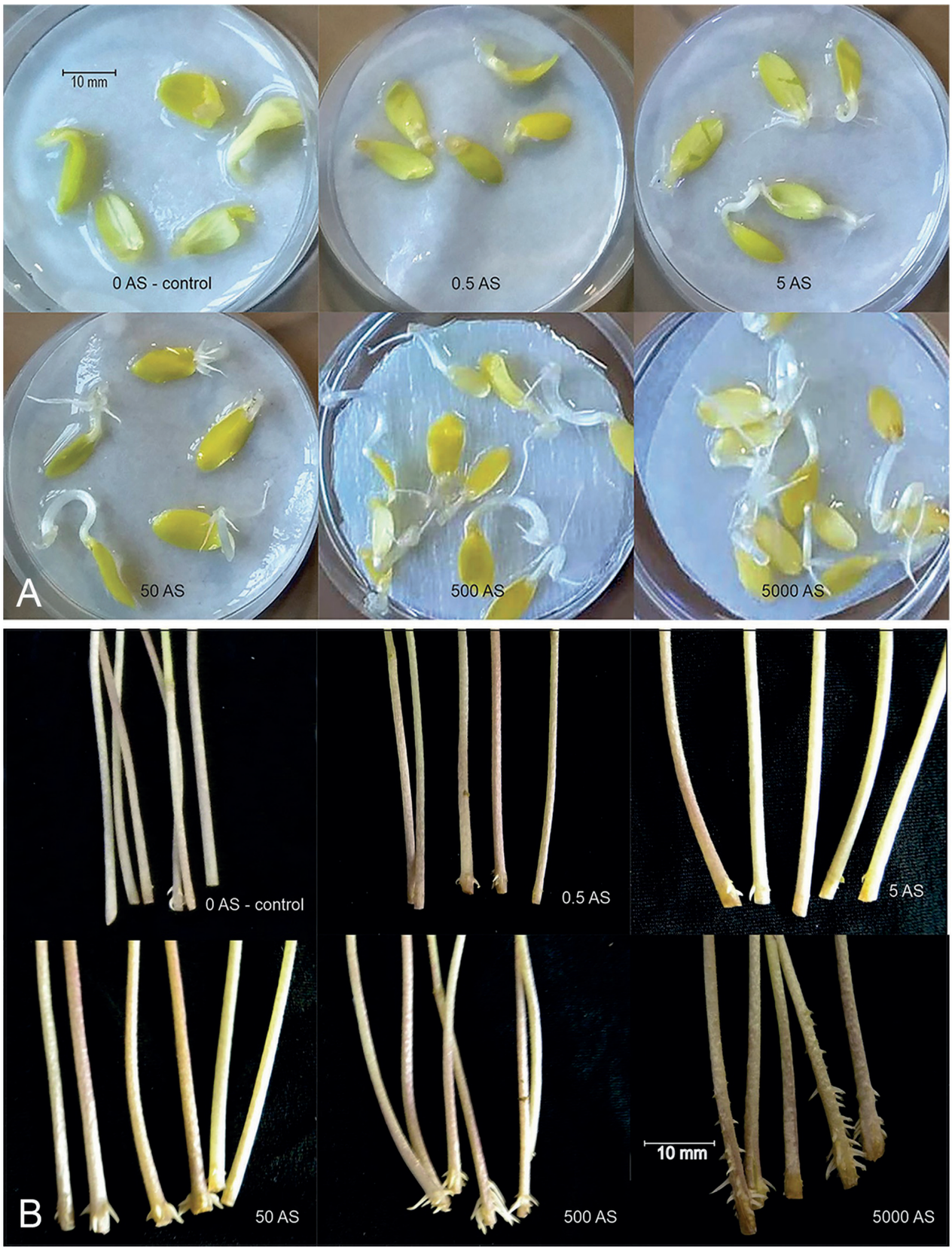

Fig. 1. The effect of Agrostemin® on root formation in (A) 'Excised cucumber cotyledon root formation bioassay' and (B) 'Mung bean root formation bioassay'.

tilled water. The vials are returned to the original growth conditions for 7 days. The solution was regularly replenished with distilled water to the original level. The number of adventitious roots, longer than 1 mm were counted at each hypocotyl after 7 days of growth and compared with the activity of the IBA control. Each treatment was replicated four times and the experiment was repeated three times.

Biological cytokinin-like activity. For cytokinin-like activity of Agrostemin® the 'Excised cucumber cotyledon expansion bioassay' (Zhao & al. 1992) and the 'Triticum leaf chlorophyll retention test' (Kühnle & al. 1977) were used.

The Excised Cucumber Cotyledon Expansion Bioassay (Zhao & al. 1992): Cotyledons for the bioassay were prepared as in the root development test described above. Cotyledons cut from 10-day-old seedlings were placed on a disk of filter paper in a 6 cm Petri dish and treated with 3 mL cytokinin, 6-benzyl-amino-purine (BAP) (0, 0.1, 0.3, 0.5, 1 and 3 mg L^{-1}), or with various dilutions of Agrostemin® (0, 0.5, 5, 50, 500, 5000 mg L^{-1}) dissolved in deionized water. Cotyledons were incubated in the dark at 25 ± 2 °C for 4 days to determine cytokinin activity. Deionized water was used as a control. The fresh weight of 10 cotyledons was recorded and compared with the activity of the different BAP concentrations. Each treatment was replicated four times and the experiment was repeated three times.

The Triticum (Wheat) Leaf Chlorophyll Retention Test (Kühnle & al. 1977): Seeds of wheat varieties (*Triticum aestivum* L.) were rinsed under running tap water for 24 hours. They were planted at a depth of 1 cm in moistened vermiculite in plastic trays and placed in the growth chamber at 25 °C in the light/dark period (16/8 h) with a light intensity of 30–60 µmol m^{-2} s^{-1} for about 7–10 days. The seeds were watered regularly. The fully expanded leaves of the seedlings (about 10 cm high) were collected and cut into 10 mm long segments 35 mm below their apical tip. The fresh weight of ten cuttings was measured with an analytical balance and placed in 25 × 90 mm glass vials (four vials per treatment) containing 10 ml of BAP (0, 0.1, 0.3, 0.5, 1 and 3 mg L^{-1}) or different dilutions of Agrostemin® (0, 0.5, 5, 50, 500, 5000 mg L^{-1}) dissolved in deionized water. Deionized water was used as control. The vials were placed back in the dark growth chamber for 4 days. After the incubation period, the leaves were dabbed dry and placed in the test tubes containing 8 ml of 80 % ethanol. The test tubes were transferred to a water bath (heated to 80–90 °C). After 10 minutes of chlorophyll extraction, the solution was cooled under running tap water and replenished to 10 ml with 80 % ethanol. Evaporation must be prevented by covering the test tubes. The cooled chlorophyll extract without the segments was then carefully poured into spectrophotometer cuvettes. The optical density (absorbance) was determined at 645 nm. The optical density was compared with 100 mg fresh weight and the adjusted results were compared with the activity of the different BAP concentrations. Each treatment was replicated four times and the experiment was repeated three times.

Statistics. The statistical package SPSS® 27.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. The level of statistical significance (p) between the different treatments was determined using Kruskal-Wallis test for independent samples. Differences at $p \leq 0.05$ were considered statistically significant.

3. Results

Agrostemin® showed auxin-like activity in the 'Excised cucumber cotyledon root formation bioassay' and in the 'Mung bean root formation bioassay', compared to IBA equivalents (Fig. 1A, 1B). The effect was already observed in a 100-fold higher dilution of 5 mg L^{-1} Agrostemin® compared to the recommended solution (Fig. 2A, 2B). However, the activity was low and comparable to less than 0.3 mg L^{-1} IBA. The effect was more pronounced in a 10-fold more dilute 50 mg L^{-1} solution, and in the recommended solution of 500 mg L^{-1}. It was also observed in a 10-fold more concentrated 5 g L^{-1} solution. The activity in the cucumber bioassay was equivalent to about 0.3 mg L^{-1} IBA and was not higher. The activity in the mung bean bioassay corresponded to about 0.5 mg L^{-1} IBA and in a 10-fold higher concentrated 5 g L^{-1} solution corresponded even to 1 mg L^{-1} IBA.

Cytokinin-like activity of Agrostemin® was determined with the 'Excised cucumber cotyledon expansion bioassay' and the 'Triticum leaf chlorophyll retention test'. Agrostemin® did not show any cytokinin-like activity when compared to BAP (Fig. 3A, 3B).

4. Discussion

It has been found that biostimulants based on algal extracts contain plant growth hormones, which are mainly responsible for stimulating plant growth and for increasing the intensity of photosynthesis (Chojnacka & al. 2012), therefore it is possible that biostimulants, based on plant preparations such as Agrostemin®, also contain plant growth regulators (PGRs) or PGR-like substances that may be responsible for stimulating plant growth. Bioassays were used to measure biological activity of the aqueous Agrostemin® solution on PGR-like responses.

In the 'Excised cucumber cotyledon root formation bioassay' and the 'Mung bean root formation

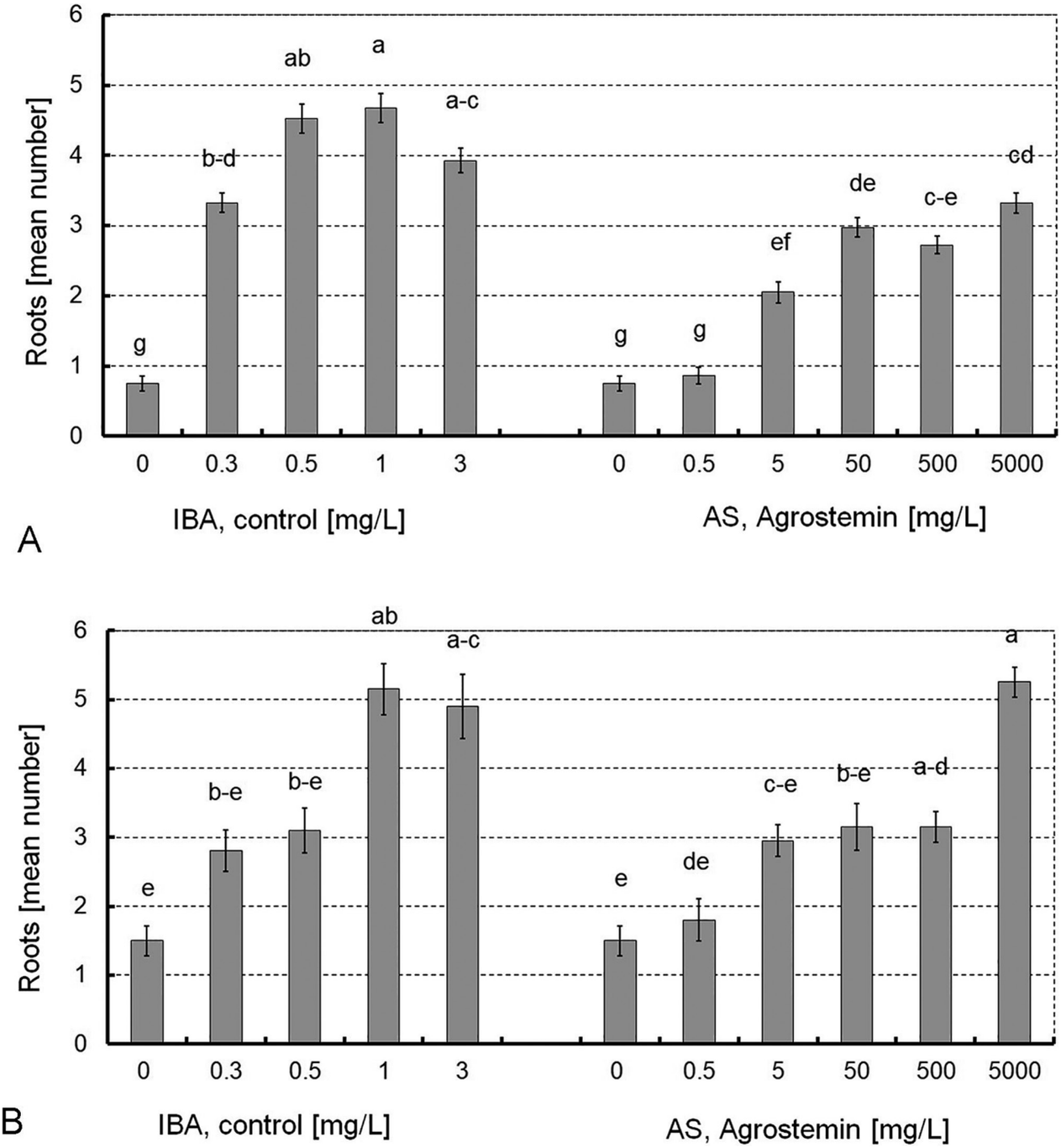

Fig. 2. Auxin-like activity detected using the (A) 'Excised cucumber cotyledon root formation bioassay' and (B) 'Mung bean root formation bioassay'. Comparison of the effect of IBA control solutions and Agrostemin® water solutions. The mean root number (n = 40) and ±SE (Standard error) were presented. Results were statistically evaluated using the Kruskal-Wallis test, followed by post-hoc comparisons. Significant differences were indicated by different letters (a–g).

bioassay', Agrostemin® showed auxin-like activity, but no cytokinin-like activity. These results indicating a stimulatory effect of Agrostemin® are consistent with the reports of Jones & van Staden (1997) and Gad & Ibrahim (2018) on algal extracts that increased rooting percentage, rooting quality, root length, and dry weight of roots in plant cuttings (Urbanek Krajnc & al. 2012).

The auxin-like substances in Agrostemin® have been shown to initiate root formation and inhibit root elongation. Plants are able to synthesize these compounds from tryptophan or indole. Root induc-

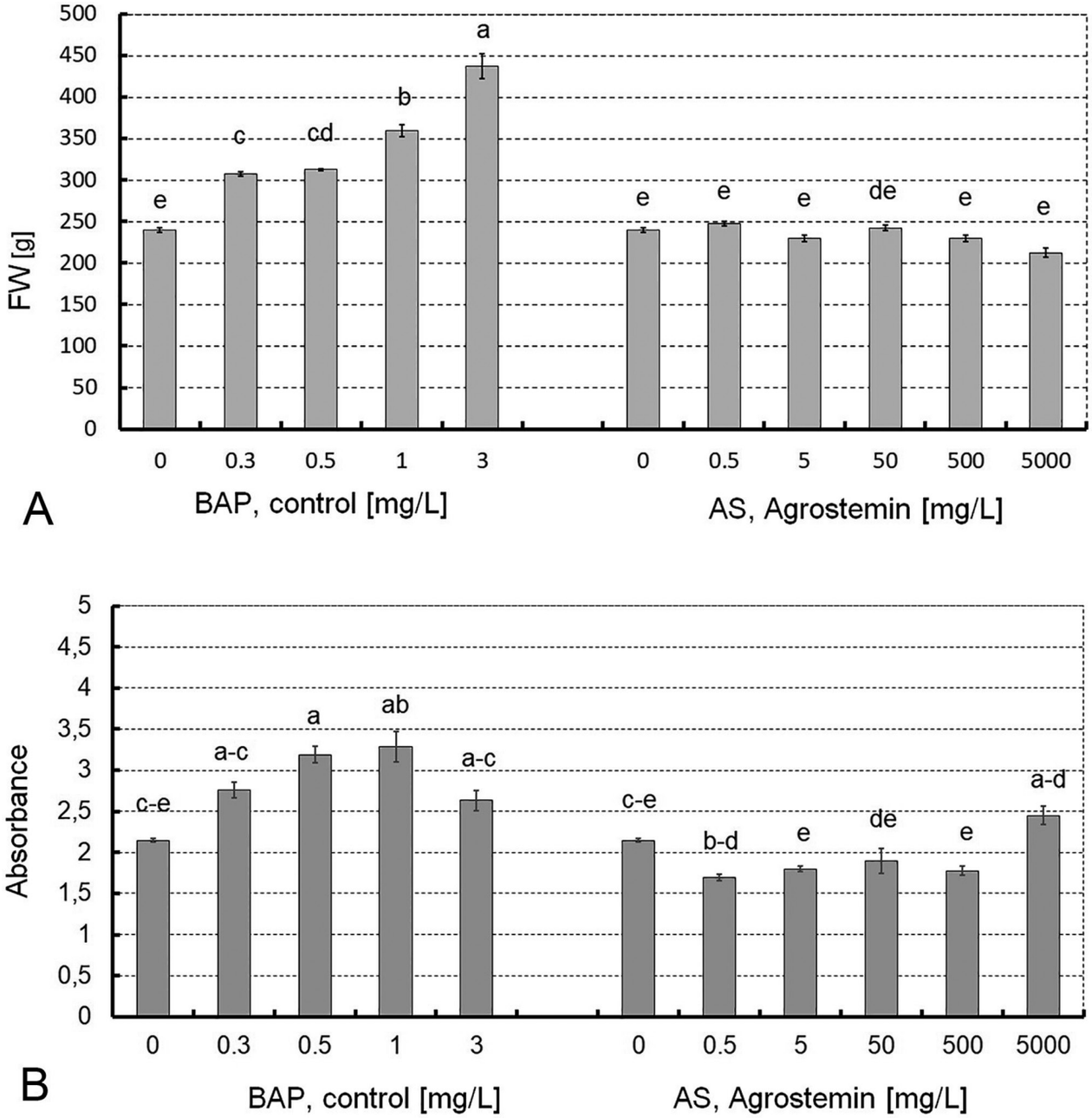

Fig. 3. Cytokinin-like activity detected using (A) 'Excised cucumber cotyledon expansion bioassay' and (B) 'Triticum leaf chlorophyll retention test'. Comparison of the effect of BAP control solutions and Agrostemin® water solutions. (A) The mean FW of 10 cotyledons (n = 40) and ± SE (Standard error) were presented, (B) the mean chlorophyll absorbance of 10 leaf segments (n = 40) and ± SE were presented. Results were statistically evaluated using the Kruskal-Wallis test, followed by post-hoc comparisons. Significant differences were indicated by different letters (a–e).

tion may be the result of a synergistic action of wounding and auxin. Wounding initiates the establishment of sink tissue for moving assimilates towards the sinks for wound-healing and cell division, while auxins induce vascular cambium differentiation and adventitious roots development (Urbanek Krajnc & al. 2012). Some other biostimulatory factors in adventitious root formation bioassays include carbon allocation and carbohydrate supply, as the number of adventitious roots formed correlates positively with sugar levels in the test material and stimulation of sink translocation towards the stem base. Auxin also inhibits leaf senescence, increases photosynthesis in leaves, and is re-

sponsible for the plasticity of shoot/root relations (Urbanek Krajnc & al. 2012). The synergistic activity can contribute to significantly higher accumulation of proteins, phenolics, and photosynthetic pigments in the leaves of *Pelargonium* cuttings. Considering the metabolic response elicited by Agrostemin®, it can be concluded that it can reduce stress following insertion into the soil and that it exhibits growth-stimulating activities, as in the Kelpak® biostimulant treatment (Urbanek Krajnc & al. 2012).

In our experimental system, we have shown that biostimulants such as Agrostemin® contain biochemical substances known as allelochemicals (Cheng & Cheng 2015), which can also be used as plant growth regulators. Their stimulatory or inhibitory role in regulating plant growth and development can be used in organic and sustainable agriculture. For example, IBA is not recommended in organic farming because it is a synthetic product (Gad & Ibrahim 2018). Therefore, the formulation of Agrostemin® must be used instead of the synthetic IBA, to promote the rooting of cuttings in nurseries. In addition to auxins, some other plant growth regulators, including salicylic acid, gibberellic acid and ethylene, which are also considered allelochemicals, can be replaced by naturally produced ones (Cheng & Cheng 2015).

The exact composition of the Agrostemin® preparation is somewhat mysterious. It is mentioned that it is made from the corn cockle (*Agrostemma githago*) and other "weed and plant" species (Agrostemin 2001, 'Prologue'). The producer also states that it consists of 39 plant species (Agrostemin 2001, 'Structure'), which are the raw and starting material for its production. These 39 plant species are not mentioned in the patent (Patent Document No. 32749, 1974). As a result, producers will have to update the registration, due to new restrictions resulting from the forthcoming regulation of this field.

Conclusion. Agrostemin® showed auxin-like activity and no cytokinin-like activity. It can be used instead of a synthetic auxin in organic and sustainable agricultural practices. With the growing demand for organic and sustainable agriculture, biostimulants are likely to continue to grow and to become, for instance, an alternative biocontrol agent.

Acknowledgements

The research was supported by the Slovenian Research Agency program 'Research for Improvement of Safe Food and Health' (ARRS, Code P1-0164) and the project 'Development of Research Infrastructure for the International Competitiveness of the Slovenian RRI Space – RI-SI-LifeWatch', co-financed by the Republic of Slovenia, Ministry of Education, Science and Sport and the European Union from the European Regional Development Fund.

References

Agrostemin 2001. Agrostemin <http://www.agrostemin.co.rs>; General Data (Structure, Formulation, etc.) <http://www.agrostemin.co.rs/opstipodacieng.php>; PRIMER – Review of application and achieved effects <https://www.agrostemin.co.rs/pdf/Web_Bukvareng.pdf>; Prologue <http://www.agrostemin.co.rs/onamaeng.php#istorijat>. – Belgrade [accessed 14 Nov 2019].

Bulgari R., Cocetta G., Trivellini A., Vernieri P. & Ferrante A. 2015. Biostimulants and crop responses: a review. – Biological Agriculture & Horticulture 31(1): 1–17.

Cheng F. & Cheng Z. 2015. Research progress on the use of plant allelopathy in agriculture and the physiological and ecological mechanisms of allelopathy. – Frontiers in Plant Science 6: 1020.

Chojnacka K., Saeid A., Witkowska Z. & Tuhy Ł. 2012. Biologically active compounds in seaweed extracts - the prospects for the application. – The Open Conference Proceedings Journal 3 (Suppl. 1-M4): 20–28.

European Biostimulant Industry Council (EBIC) 2016. <http://www.biostimulants.eu/> [accessed 23 Feb 2016].

Gad M. M. & Ibrahim M. M. 2018. Effect of IBA and some natural extracts on rooting and vegetative growth of Picual olive sucker and shoot cuttings. – Current Sciences International 7(2): 191–203.

Hess C. E. 1961. The mung bean bioassay for detection of root promoting substances. – Plant Physiology 36(I): suppl. 21.

Jones N. S. & van Staden J. 1997. The effect of a seaweed application on the rooting of pine cuttings. – South African Journal of Botany 63(3): 141–145.

Kühnle J. A., Fuller G., Corse J. & Mackey B. E. 1977. Antisenescent activity of natural cytokinins. – Physiologia Plantarum 41(1): 14–21.

Mangotić N. 2003. Stemini – bioregulatorji prihodnosti (Stemins – bioregulators for the future). – Zbornik predavanj in referatov. 6. Slovenskega Posvetovanje o Varstvu Rastlin. Zreče, Slovenije, 4–6 marec 2003, p. 335–338.

Patent Document No. 32749, 1974. A process for stimulation of growth and development of "allantoin" type plants, 1974. – Federal Commission for Patents, SFR Yugoslavia; Belgrade.

Povero G., Mejia J. F., Di Tommaso D., Piaggesi A. & Warrior P. 2016. A systematic approach to discover and characterize natural plant biostimulants. – Frontiers in Plant Science 7: 435.

Stirk W.A., Bálint P., Tarkowská D., Novák O., Maróti G., Ljung K., Turečková V., Strnad M., Ordög V. & van Staden J. 2014. Effect of light on growth and endogenous hormones in *Chlorella minutissima* (*Trebouxiophyceae*). – Plant Physiology and Biochemistry 79: 66–76.

Urbanek Krajnc A., Ivanuš A., Kristl J. & Šušek A. 2012. Seaweed extract elicits the metabolic responses in leaves and enhances growth of *Pelargonium* cuttings. – European Journal of Horticultural Science 77(4): 170–181.

Yakhin O. I., Lubyanov A. A., Yakhin I. A. & Brown P. H. 2016. Biostimulants in Plant Science: a global perspective. – Frontiers in Plant Science 7: 2049.

Zhao Z., Wu Z., Huang G. & Li G. 1992. An improved disk bioassay for determining activities of plant growth regulators. – Journal of Plant Growth Regulation 11(4): 209–213.

(Received 28 May 2021, accepted 13 Dec 2021)