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Bioactivity of *Scytonema hofmanni* (Cyanobacteria) in *Lilium alexandrae in vitro* propagation

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Keywords: ascorbate peroxidase, bioactive substances, bulb regeneration, catalase, Cyanobacteria, glutathione reductase, *Lilium alexandrae, Scytonema hofmanni*.

Abbreviations: BWE: biomass water extract.
EP: extracellular products.
NAA: naphthalene acetic acid.
MS: Murashige-Skoog.

Cyanobacteria produces bioactive compounds including plant growth regulators. Naphthalene acetic acid (NAA), a toxic substance, is a synthetic plant regulator used in micropropagation. The aim of this work was to evaluate morphogenetic and antioxidant effects produced by intra and extracellular substances from *Scytonema hofmanni* (Cyanobacteria) during the multiplication *in vitro* of *Lilium alexandrae* and to compare them to those produced by NAA. Intra and extracellular cyanobacterial products increased a) bulblets production reaching 83% and 78% of NAA effect, respectively; b) the bulblet diameter compared to NAA; and c) the bulblet survival due to the promotion of antioxidant activity measured as catalase, ascorbate peroxidase, and glutathione reductase activity. The cyanobacterial substances stimulated regeneration and delayed bulblet senescence. They could replace NAA, dangerous for the operator, not only during the regeneration phase but also during the storage of the viable bulblets cultivated *in vitro*.

Growth regulators which accelerate the production of a number of agronomical interesting plants are used in *in vitro* culture of plants. Growth regulators are mainly obtained by chemical synthesis. Cyanobacteria produce a variety of bioactive compounds including growth phyto-regulators (Metting and Pyne, 1986), that could be used in the *in vitro* production of vegetables, fruits, fungi and ornamental flowers. Already in 1979, Zulpa de Caire et al. (1979) established that *Nostoc muscorum* Ag. liberated into the culture medium auxin-like substances. It has recently demonstrated that a number of Cyanobacteria produce, accumulate, and liberate 3-indol acetic acid (Sergeeva et al. 2002). *Arthronema africanum* produces the cytokinin isopentenyl adenine (Stirk et al. 1999). Stirk et al. (2002) found auxin and cytokinin activity by three cyanobacterial strains. It is important to mention that some cyanobacterial products promote regeneration in *Daucus carota* (Wake et al. 1992), *Santalum album* (Bapat et al. 1996), *Oryza sativa* (Zaccaro et al. 2002; Storni de Cano et al. 2003), *Lilium alexandrae* hort. Wallace, from Japan, is an ornamental plant, with white flowers bending down to form an angle of 90º, with respect to the vertical axis of the plant making this species a very interesting one for the improvement of other *Lilium* species (Nakayama, 1989). Naphthalene acetic acid (NAA) is a synthetic phyto-regulator very much used in micropropagation. Toxicological information indicates that NAA causes contact irritation of skin, eyes, mucous membranes, as well as the upper respiratory tract. It also attacks the central nervous system and can be absorbed by the operators skin (Sigma – Aldrich-Argentina S.A.). The aim of this work was to evaluate the morphogenetic and antioxidant effects produced by intra and extracellular substances from *Scytonema hofmanni* (Cyanobacteria), on the *in vitro* propagation of *L. alexandrae* and to compare them with those produced by synthetic phyto-regulators.

MATERIALS AND METHODS

Explant obtention

Microscales (6-8 x 6-10 mm) from bulblets of *L. alexandrae* (1.8-2.5 g fresh weight and 5-6.2 cm diameter), were obtained *in vitro* after 120 days growing on MS medium (Murashige and Skoog, 1962), with a photoperiod

Table 1. Activity of antioxidant enzymes in bulblets at day 260. Different letters indicate significant differences ($p < 0.05$).

Treatment	Catalase (umol/min g fresh weight)	Ascorbate peroxidase (umol/min g fresh weight)	Glutathione reductase (umol/min g fresh weight)
MS	7.212 ± 0.003 [d]	10.604 ± 0.010d	2.212 ± 0.700C
MS + NAA	15.511 ± 0.003[c]	44.051 ± 0.005a	3.384 ± 0.050C
MS + EP	59.852 ± 0.005[a]	16.784 ± 0.005c	14.034 ± 0.060A
MS + BWE	24.893 ± 0.004[b]	32.623 ± 0.005b	5.854 ± 0.050B

of 12 hrs light and a light intensity of 45 µmol photon x m^{-2} seg^{-1} and 25 ± 1ºC. The abaxial side of the explant was placed on the culture medium.

Obtaining of cyanobacterial products

Biomass water extracts (BWE) and extracellular products (EP*).* *S. hofmanni Ag*. ex Born. et Flahault axenic strain Nº 58, from the culture collection belonging to Laboratorio de Cyanobacteria, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina, was cultivated in modified Watanabe medium (Storni de Cano et al. 2003), photoperiod 12 hrs light, 45 µmol photon x m^{-2} seg^{-1}, 27 ± 1ºC. After 30 days growing in that medium, the biomass was separated from the culture medium by centrifugation at 10.000 xg at 5ºC. The fresh biomass was homogenized with alumina (1 g biomass / 3 g alumina), and extracted with sterile distilled water (1 g fresh biomass / 5 ml water). After centrifugation the supernatant BWE was obtained. The culture medium contained the extracellular products (EP). BWE and EP were sterilised by ultrafiltration (0.22 µm).

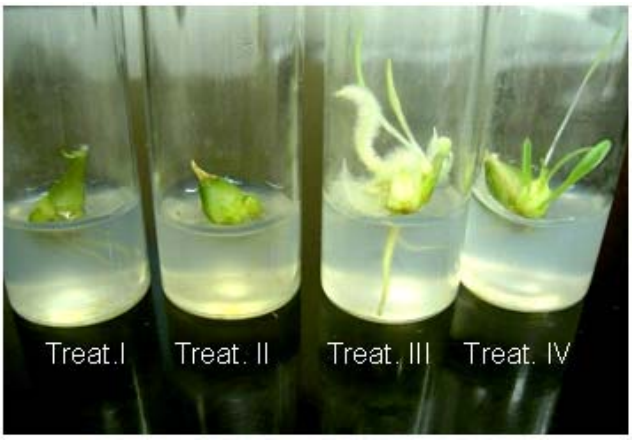

Figure 1. Bulblets, leaves and roots differentiation in micro scales from *L. alexandrae.* Treatments: I (MS), II (MS + EP), III (MS + NAA) and IV (MS + BWE), at day 50.

Obtaining of bulblets

Treatments: **I**, 8 ml MS + 2 ml distilled water. **II**, 8 ml MS + 2 ml EP. **III**, 8 ml MS + 2 ml distilled water + 0.1 mg/L NAA. **IV**, 8 ml MS + 2 ml BWE. The media contained 3% sucrose, 0.7% agar and pH was adjusted to 6.8, before being sterilized in tubes (14 x 2.5 cm). The explants were placed in a dark culture chamber, for three weeks in order to obtain differentiation and bulblets growth. After that period they were transferred to 16:8 photoperiod, 45 µmol photon x m^{-2} seg^{-1} and 25 ± 1ºC. Each 5 weeks, they were transferred to fresh medium. At days 20, 30, 40, 50 and 70 the number of bulblets per explant, bulblet longest diameter, bulblet roots and leaves length and number per explant were measured. At day 260 in this case without transference to fresh medium, the survival capacity of the bulblets in each treatment was established.

Biochemical parameters

Enzymatic activity of catalase, ascorbate peroxidase and glutathione reductase were determined according to Beers and Sizer (1951), Nakano and Asada (1981) and Schaedler and Bassham (1977), respectively, in order to establish the bulblets oxidative stress level produced by the regeneration treatments.

Statistic analysis

Analysis of variance was performed for all data, using a completely randomized experimental design. A one-way ANOVA was performed (PC program GraphPad Prism). A Tukey HDS test ($p < 0.05$) was used to compare different treatments for each day, $n = 14$.

RESULTS AND DISCUSSION

At day 50 (Figure 1) *S. hofmanni* BWE increased the number of bulblets per explant by 27% comparing with MS, representing this increment 78% of the bulblets produced by NAA. At day 70 the increment was 21 y 32% for EP and BWE, respectively, compared to MS. With respect to NAA, EP produced 78% and BWE 83% of the number obtained with the synthetic auxin (Figure 2a). No matter higher the number of bulblets produced by NAA, cyanobacterial intracellular products increased the number of bulblets thicker than 5 mm as at day 70 BWE surpassed NAA, MS and EP effect by nearly 30% (Figure 2b). This effect on thickness is important to survival of the bulblets. The number of roots per explant (Figure 2c) in MS and EP was lesser than NAA by 70% and in BWE by 50%, at day 70. This could mean that BWE favoured the higher thickness of the bulblets, compared to NAA, at the expense

of the number of roots, probably due to a differential distribution of organic nutrients. The number of leaves per explant (Figure 2d) was the same in EP and NAA at day 70, showing at the beginning an acceleration of differentiation with EP. Extracellular products from other Cyanobacteria were shown to replace synthetic phyto-regulators in the organogenesis of rice calli (Storni de Cano et al. 2003), to increase the phyto-regulators content of *Lupinus termis* (Haroun and Hussein, 2003). Cyanobacterial crude extracts showed auxinic activity in potato tissue culture (Shanab et al. 2003). At day 260 the number of bulblets decreased in all the treatments. In MS 50% bulblets were necrotic and in NAA 100%, but with EP and BWE 90% were viable. Table 1 shows that EP and BWE produced an increment of 3.85 and 1.60 fold in catalase activity with respect to NAA which in turn doubled this activity with respect to MS. Bulblets ascorbate peroxidase activity increased 4 times with NAA, 3 times with BWE and 1.67 times with EP. Glutathione reductase activity increased 6.34 times with EP, 2.64 times with BWE and 1.52 times with NAA comparing with the control with MS. The activity of enzymes related with reactive oxygen species was remarkably incremented by cyanobacterial products, surpassing NAA for catalase and glutathione reductase activity which could explain the higher survival of bulblet that were not transferred to fresh medium for 260 days remaining viable by 90%. It is known that the activity of antioxidant enzymes increases in tissues under different stress conditions, such as sugar beet calli (Hagége, 1996).

CONCLUDING REMARKS

Intra and extracellular products from *S. hofmanni* increment *L. alexandrae* bulblets production from microscales, comparing with control without synthetic phyto-regulators as well as bulblets survival in culture because they promote a higher antioxidant activity. This effect could be due to the production and liberation of plant growth regulators such as

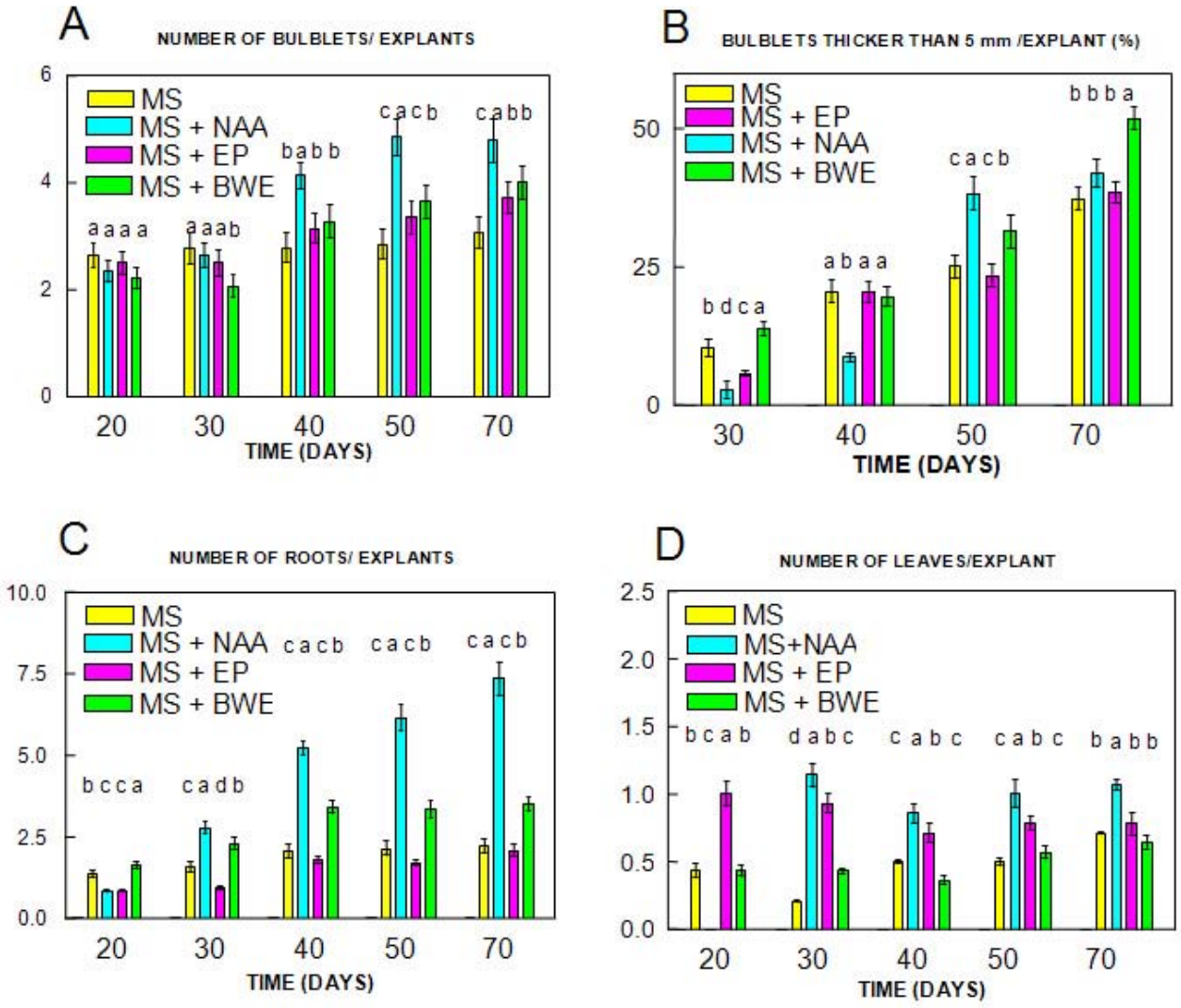

Figure 2. Morphogenetic development of explants from bulblets of *L. alexandrae*. Different letters indicate significant differences ($p<0.05$).

auxin-like and cytokinin-like substances by the cyanobacterium, which produced similar results than those obtained with the synthetic auxin NAA and also because the bulblets senescence was delayed, an effect well-known for cytokinin. Besides, the cyanobacterial phyto-regulators could replace substances that result dangerous to the operator not only during the regeneration phase but also during the storage of the viable bulblets cultivated *in vitro*.

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