Seed biopriming with cyanobacterial extracts as an eco-friendly strategy to control *damping off* caused by *Pythium ultimum* in seedbeds

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Abstract

This work highlights the ability of various cyanobacterial extracts from *Anabaena* spp., *Tolypothrix* spp., *Nostoc* or *Trichormus*, among others genera, to control the incidence of *damping-off* caused by *Pythium ultimum* in cucumber seedlings. Protocols applied aimed at the preliminary characterization of the cyanobacterial collection were very useful for predicting their phytotoxic, phytostimulating and biopesticidal capacity. First, the phytostimulatory or phytotoxic potential of a collection of 31 sonicated cyanobacterial extracts was analyzed by calculating the germination index in watercress seeds and the increase or loss of seedling weight. Likewise, the collection was characterized according to its ability to inhibit the growth of *P. ultimum* by dual culture bioassays and detached-leaf test. Finally, after selecting the most effective extracts, a preventive *damping-off* bioassay was performed based on cucumber seed biopriming. The strain SAB-M465 showed to be the most efficient

strain against the *in vitro* growth of *P. ultimum*, while SAB-B912 was more discreet in this regard, but proved to be the most effective as a germination stimulator. Seed biopriming strategy with sonicated extracts of cyanobacteria revealed a remarkable promoter effect in the early stages of plant development, although only SAB-M465 was positioned as an effective control agent against *damping-off* caused by *P. ultimum* in cucumber seedbeds.

Keywords: Cyanobacteria; plant protection; biopesticides; phytotoxicity; biopriming; *damping-off*.

Highlights

- Cyanobacteria distinguished as biological control agents in seeds and seedlings
- SAB-M465 stood out as a muting agent for *damping-off* in seedbeds
- SAB-B912 stimulated plant growth in pre and post emergence phases
- Seed *biopriming* was successful as a preventive treatment against *damping-off*
- Seed *biopriming* was positioned as an eco-friendly strategy to promote plant growth

1. Introduction

The practice of intensive agriculture has traditionally been related to the application of chemical products that are highly polluting for the environment and sometimes dangerous for health. In order to guarantee consumer and environmental health, a sustainable and eco-friendly control of diseases affecting the early stages of plant growth is essential (Bourguet and Guillemaud, 2016; Lamichhane *et al.,* 2016).

Cucumber (*Cucumis sativus L*.) is one of the most important vegetable crops growing all over the world in open fields, tunnels and greenhouses (Soleimani *et al*., 2009). Specifically, in the southeast of Spain, cucumber cultivation, together with tomato and pepper, is one of the main crops in terms of greenhouse production and export to other European Union countries (ARG, 2021). However, cucumber seedlings are susceptible to a wide range of pathogens. This fact leads to significant economic losses derived from the lack of quality and yield of cucumber crop under favourable conditions for disease development (Georgakopoulos *et al*., 2002; Abbasi and Lazarovits, 2006).

In terms of diseases caused by phytopathogenic agents, *damping-off* is one of the most widely spread. A number of soil-borne pathogenic fungi have been associated with pre- or post-emergence *damping-off*. That is the case of different species of *Pythium*, *Phytophthora*, *Fusarium* and *Rhizoctonia* (Lamichhane *et al*., 2017). Although there is no detailed and precise estimation about the real economic impact of *damping-off* at the

global level in monetary terms, an extensive literature research showed that the incidence of damping-off may vary from 5 to 80%

(Lamichhane et al., 2017; Liu *et al.*, 2020). The oomycete *Pythium ultimum* is an important soil-borne plant pathogen that causes *damping-off* on over 300 diverse plant species including cucumber and other cucurbits (Kamoun *et al.,* 2015). These pathogens are usually polyphagous and there is no natural resistance in plants to their infections. However, controlling *damping-off* with antagonistic microorganisms can be relatively easy, if they are preventively used. In other words, antagonists need to be introduced before the potential entry of the pathogen (Li *et al*., 2016).

The most common bacterial antagonistic agents that have been traditionally used to control *damping-off* include several species of the genera *Bacillus* and *Pseudomonas* (Georgakopoulos *et al.,* 2002; Carisse *et al.,* 2003). Nevertheless, cyanobacteria are also globally gaining attention as plant growth promoting and biocontrol agents in diverse crops including rice, wheat, cotton, legumes, solanaceae and cucurbitaceae (Prasanna *et al*., 2009; Manjunath *et al*., 2010; Prasanna *et al*., 2015).

The inoculation of these organisms influences various metabolic processes in plants, since they activate the production of defense proteins that lead to a greater immunity of plants against pathogens (Gupta *et al*., 2013). On the other hand, it is known that the inoculation of cyanobacteria directly in the soil or by seed immersion or *priming* causes an increase in the germination rate, a better development of plants and a higher productive yield in a wide variety of cereal, horticultural and

vegetable crops (Rodríguez *et al*., 2006; Saadatnia and Riahi, 2009).

One of the newest methods based on seed conditioning by treatment with beneficial microorganisms, *biopriming,* is gaining wide acceptance (Anitha *et al*., 2013). This preventive method aims to achieve a durable and effective impact of the beneficial microorganisms. In this way, it ensures that the physiological processes, due to interaction seed-microorganism, provide the seed with the desired capabilities with respect to plant biostimulation and plant protection against pathogens (Harris *et al*. 2008). There are numerous researches from which the effectiveness and survival of the applied agents has been proven by the described technique, ensuring the optimal plant yield and growth (Yadav *et al*., 2018). Nonetheless, in the particular case of cyanobacteria, the work is still very incipient.

The aim of this work was to investigate the potential bioprotective effect from a cyanobacterial collection against the *damping-off* caused by *Pythium ultimum* in cucumber seedbeds. Specific objectives were: (i) to check the phytotoxic or phytostimulant effect of the treatment with cyanobacterial extracts on the germination of watercress seeds; (ii) to examine the *in vitro* efficacy of different cyanobacterial strains on the inhibition of mycelial growth of *P. ultimum*, by dual culture technique, as well as by detached cucumber-leaf bioassays, and (iii) to investigate both biostimulant and biopesticide impact *in vivo* by a *biopriming* preventive assay in cucumber seeds. This study will lay the foundations for future research on the utilization

of cyanobacteria not only in its widely known application as biofertilizer, but also in managing crop diseases.

2. Material and Methods

2.1 Microorganisms

2.1.1 Phytopathogenic agent

An active culture of the phytopathogenic oomycete *Pythium ultimum* CECT 2365 (PU) were supplied by the Spanish Type Culture Collection (STCC). Cultures of the phytopathogenic agent were periodically kept on Potato Dextrose Agar (PDA, Oxoid Ltd., UK) at 4 °C until use.

2.1.2 Collection of cyanobacteria and getting extracts

A total of 31 strains of cyanobacteria were studied in this work. Strains were supplied in lyophilized form from two international culture collections: Mosonmagyaróvár Algal culture collection (MACC) and Spanish Bank of Algae (SBA). The collection included species of the genera *Anabaena, Calothrix Cyanobacteria, Dolichospermum, Gloeocapsa, Leptolyngbya, Lyngbya, Nodularia, Nostoc, Phormidium, Synechococcus, Tolypothrix*, and *Trichormus*. Before starting all *in vitro* and *in vivo* tests, lyophilized biomass from each strain was subjected to a quick sonication process (Branson Sonicator 150) at 40% amplitude for 3 minutes, to obtain liquid extracts at a stock concentration of 10 mg mL^{-1}.

2.2 *In vitro* plant growth promotion bioassays

2.2.1 Root lengthening promotion: Germination Index

Promotion effect on germination and radicular elongation was tested on four replicates of 25 seeds for each concentration of cyanobacterial extract prepared (2 and 0.5 mg DM mL^{-1}) (DM: dry matter). To calculate Germination Index, percentage of seed germination and elongation of the radicle (mm) were taken into account, based on the following formula (Zucconi, 1981):

GI= (Ge% * REe) / (Gdw% * REdw)

Where:

GI: Germination Index (%)

Ge%: percentage of germinated seeds in presence of extract

Gdw%: percentage of germinated seeds in distilled water

REe: mean of radicle elongation (mm) in presence of extract

REdw: mean of radicle elongation (mm) in distilled water

2.2.2 Seedling weight after emergence

Weight of the seedling was tested on 100 watercress seeds at concentrations of extract 2 and 0.5 mg DM mL^{-1}, by the modified technique described by Hegab *et al*., (2008). Germination of the watercress seeds was carried out by using four replicates of 25 seeds for each concentration of cyanobacterial extract prepared (2 and 0.5 mg DM mL^{-1}. The fresh weight measurement (expressed in mg) of the 25 seedlings of each Petri dish was recorded.

The increase of seedling weight (ISW) was calculated on the basis of the following formula:

ISW= (Ge% * SWe) / (Gdw% * SWdw)

Where:

ISW: Increase of Seedling Weight (%)

Ge%: percentage of germinated seeds in presence of extract

Gdw%: percentage of germinated seeds in distilled water

SWe: mean of seedling weight (mg) in presence of extract

SWdw: mean of seedling weight (mg) in distilled water

2.3 *In vitro* antagonism bioassays towards Pythium *ultimum*

2.3.1 Dual culture protocol

Once the cyanobacteria extracts were sonicated (see section 2.1.2.), their biological activity was tested against PU by dual cultures following the protocol described by Sánchez-San Fulgencio *et al*. (2018).

Suppressive effect was demonstrated using the modified techniques of Landa *et al*. (1997). First, 2% water agar (WA) plates were prepared. Once the agar solidified, four 8-mm-diameter steel cylinders were placed equidistantly from the edge. A second layer of PDA was added on the WA plates. Once the cylinders were removed, the wells were filled with 80 µL extract of the cyanobacteria at stock concentration of 10 mg mL^{-1}. Then, a plug of 5-day-old phytopathogen agent culture, removed from a PDA plate, was placed at the center of the assay plate. Four

replicates were prepared for each combination extract-phytopathogen. *In vitro* growth inhibition of PU was measured at 25 ± 1 °C under dark conditions after 2 days. The inhibition index (I) was expressed as the percentage of PU inhibition in the presence of the antagonistic strain using the formula:

$I = [(C - T)/C] \times 100$

Where:

I: Inhibition Index (%)

C: growth of the phytopathogenic agent in absence of the antagonistic strain (mm)

T: growth of the phytopathogenic agent in presence of the antagonistic strain (mm).

2.3.2 Detached leaf bioassays

This bioassay was carried out modifying the method described by Guo *et al*. (2015). For this purpose, young cucumber leaves were cut and collected from seedlings after one month from emergence. Inside an empty and sterile Petri dish, the detached cucumber leaves were completely immersed for 10 seconds in 20 mL of the cyanobacterial extracts (0.1 mg mL^{-1}). After immersion, the leaves were placed inside Petri dishes with sterile filter paper moistened with 4 mL of sterile distilled water. Then, a mycelial plug (approximately 5 mm in diameter) from active growing margins of a 2-day old PDA culture of PU, was placed in the center of each cucumber leaf. Negative control leaves were immersed in sterile distilled water with a plug of PDA without

mycelium. Petri dishes were sealed and incubated at 25 ± 1 °C for 6 days under dark conditions. The diameter of the infected area of each leaf were measured at 2, 4 and 6 days after incubation. Disease rating was expressed as leaf lesion diameter (mm) regarding lesion observed in negative control leaves non-previously treated with the cyanobacterial extracts. Five replicates were prepared for each foliar treatment.

2.4 *In planta* bioassays

2.4.1 Cucumber seed bioimmersion protocol

Cucumber seeds were washed with sterile distilled water 3 times before being subjected to the immersion bioassays. Then, they were immersed for one hour in 10 mL volume containing a semi-solid matrix constituted by 0.5% sodium alginate combined with the sonicated microbial extracts selected on the basis of previous experiments at 0.1 mg DM mL^{-1}. Thirty replicates were prepared for each seed *biopriming* treatment.

2.4.2 Substrate infestation with a zoospore suspension of *Pythium ultimum*

A stock culture of PU was subcultured onto 15 Pea agar medium plates (Tello *et al*., 1991). Each plate was inoculated with a 4 mm diameter plug of mycelium taken from the edge of an actively growing culture on PDA. Cultures were incubated at 25 °C for one week in the darkness.

After incubation, mycelium from each plate was cut in two halves and one half was placed in other empty sterile Petri dish. Both halves were rinsed with sterile distilled water, and then

incubated in the same conditions previously described. Release of zoospores was favored by thermal shock. Thereby, cultures were maintained at 4 °C for one hour, followed by another hour at room temperature. Finally, the culture surface was scraped with a sterile spatula. In order to remove the culture debris, the resultant zoospore solution was then filtered through a sterile cheesecloth layer. Zoospore concentration was determined by Neubauer chamber count and then diluted to a suspension of 10^5 zoospore mL^{-1}.

Pots filled with sterilized substrate (mix peat:vermiculite at 3:1 ratio) were infected at the rate of 10^5 zoospore / pot and covered with cling film to maintain the humidity conditions for one week until the seeds were sown.

2.4.3 Protective and phytostimulant effect of cyanobacteria extracts towards *damping-off* caused by *Pythiun ultimum* in cucumber seeds after *biopriming*

The most effective cyanobacterial extracts selected from preliminary experiments were *in vivo* assayed to determine their potential as plant growth promoters of seedlings and for their protective effect against PU in *bioprimed* cucumber seeds. A randomized experimental design was established consisting of pretreated cucumber seeds as previously described in section 2.4.1. Pretreated seeds were sown into infected substrate with PU as described in section 2.4.2. Seeds immersed in sterile distilled water and germinated in uninfected soil were used as negative control. Plants were kept in a climatic chamber (EQUITEC Mod. ERIS 615) at a temperature of 25 ± 1° C and a 12 h photoperiod.

After 10 days from sowing, *damping-off* was evaluated by measuring percentage of germination in the different experimental blocks respecting to control seeds submerged in sterile distilled water. *Damping-off* was considered when the seed did not germinate or when it died just after the emergence. On the other hand, phytostimulant effect was evaluated 28 days after sowing, as previously described by Santoro *et al*. (2011). Thereby, the following parameters were measured to evaluate the plant vigor: stem and root length (cm), stem diameter (mm), fresh weight (g) and ratio root/stem.

2.5 Statistical analyses

Data obtained were subjected to statistical analysis using Statgraphics Centurion XVII. A multifactorial analysis of variance (ANOVA) and a multiple comparison test (Fisher's Least Significant Difference) were performed to compare mean values for different levels of repetition ($P < 0.05$). A linkage between groups was used as a grouping method for the variables analyzed *in vitro*. The interval measured in this case was the nearest Euclidean distance squared.

3. Results and Discussion

3.1 Growth-promoting effect on germination index (GI) and increase in seedling weight (ISW)

A collection of 31 cyanobacteria was evaluated to study the phytotoxic or phytostimulant effect *in vitro* from cyanobacterial extracts applied in watercress seeds (Fig. 1). For that purpose,

two determinations were carried out. On the one hand, Germination Indexes (GI) based on the percentage of germination of watercress seeds and the radicle elongation (Figs. 1a,b), were calculated. On the other hand, Increase of Seedling Weight (ISW), based on the percentage of germination of seeds and the fresh weight of the radicles after emergence was also estimated (Figs. 1c,d).

Regarding Germination Index, those strains which turned around or exceeded 100% GI were considered as potential phytostimulant agents. When aqueous extracts were applied at the concentration of 0.5 mg mL^{-1}, strains SAB-M128, SAB-M612, SAB-M683, SAB-B912, SAB-B1211, SAB-B1269, SAB-B1572, SAB-B1328, SAB-B880, SAB-B450 and SAB-B786 stood out as potential plant growth promoter cyanobacteria (Fig. 1a). Some of the mentioned strains also showed a phytostimulant effect at a higher concentration (2.0 mg mL^{-1}); this was the case of the strains SAB-M683, SAB-B912, SAB-B1211, SAB-B1328 and SAB-B880. It should be noted that GI values passed 120% when the extracts from the strains SAB-B912 and SAB-B1211 were applied at 2.0 mg mL^{-1}. Contrary to what was stated above, SAB-M304 and SAB-B1579 were especially phytotoxic, producing values below 25% of germination index (Fig. 1a).

The distribution of the results here obtained revealed a slightly negative trend as the concentration of the extracts applied in this test was increased (Fig. 1b). In fact, based on the box and whisker diagram shown in Figure 1b, near of 100% of the cyanobacteria extracts applied at 0.5 mg mL^{-1} were positioned in GI values between

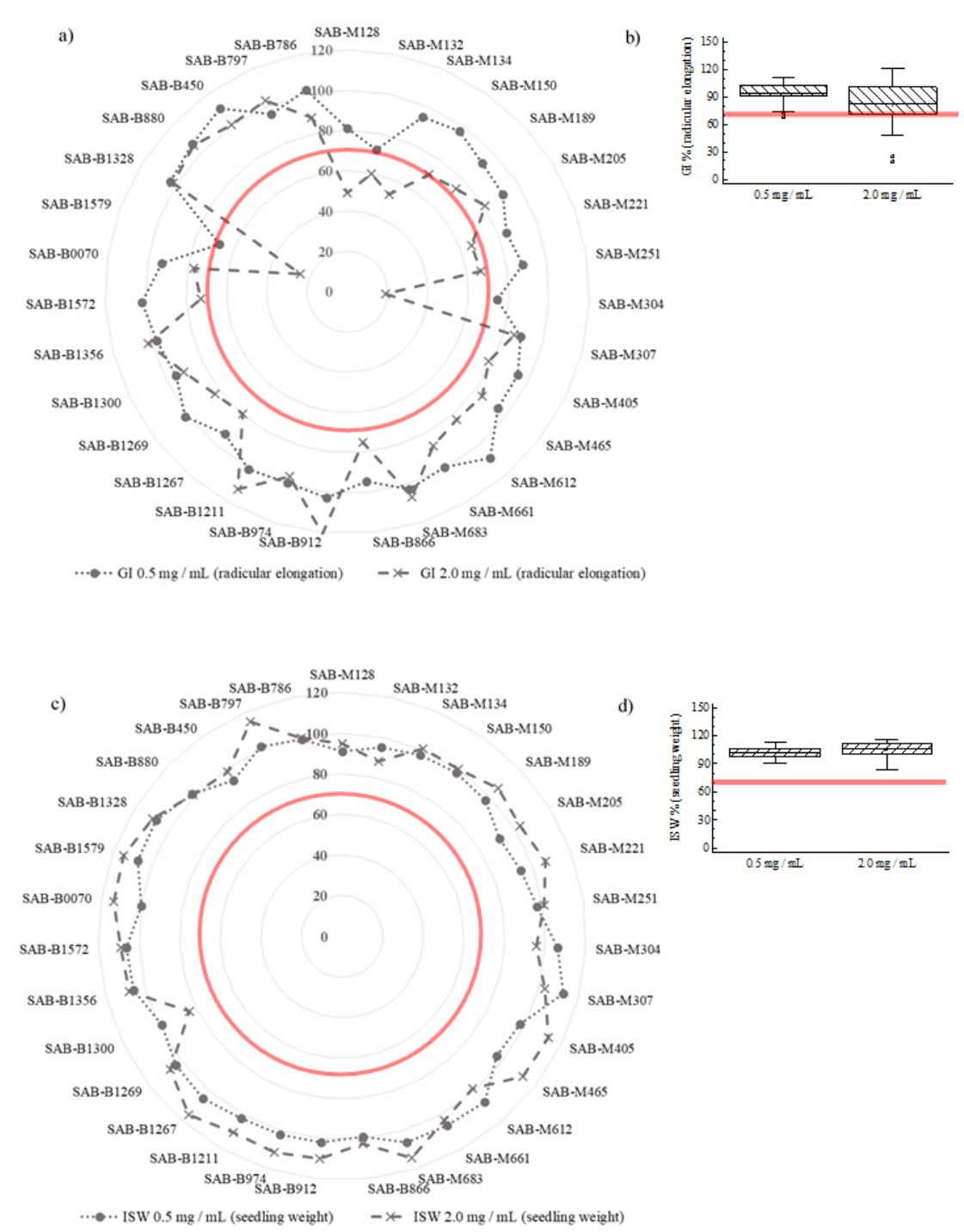


Figure 1. Biostimulant or Phytotoxic effect derived from extracts of cyanobacteria sonicated by bioassays in watercress seeds: a-c) Mean of Germination Index (GI%) and Increase of Seedling Weight (ISW%) produced by each cyanobacterial extracts at two different concentrations (0.5 and 2.0 mg mL-1); b-d) Box and Whisker Plots showing central tendency and dispersion of GI and ISW at two different concentrations (0.5 and 2.0 mg mL-1). 100 watercress seeds were used

for each of the bioassays carried out; red line indicates the GI (%) and ISW (%) value below which the extracts were considered phytotoxic.

70-115%, showing an average value of GI higher than 90%. In contrast, the results derived from the extracts applied at 2.0 mg mL^{-1} followed a more scattered distribution. In this sense, 75% of the strains showed a GI between 70-120%. Remaining 25% belonged to potentially phytotoxic extracts, showing a GI range between 25-70% (Fig. 1b).

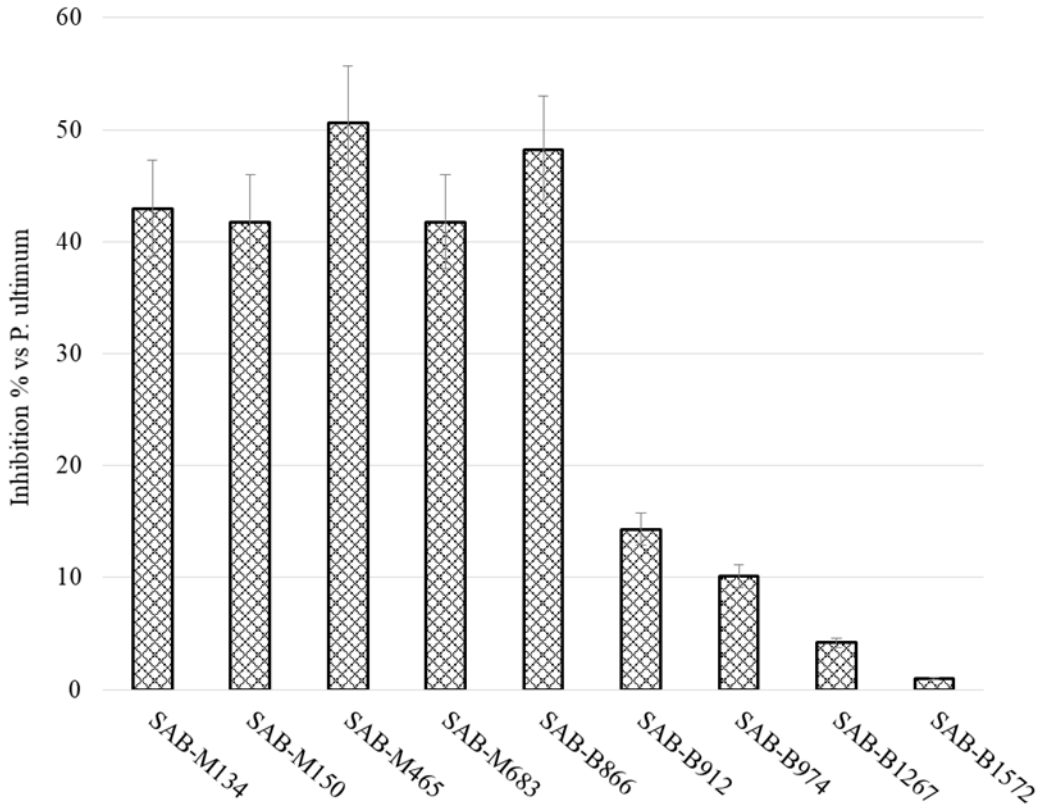
Regarding the weight increase of the germinated radicles (ISW), similarly to what has been considered for GI, strains that were around or exceeded 100% in this parameter were considered potentially phytostimulant, provided they were not phytotoxic with respect to root lengthening. As seen in Figures 1c and 1d, the distribution of the data was very homogeneous with respect to the application of both concentrations of extract. The dispersion of the ISW data was somewhat less in the case of extracts applied at 0.5 mg mL^{-1}, ranging from 90-115%, while it was somewhat greater when the extracts were applied at 2 mg mL^{-1} (Fig. 1d). The average mean ISW was above 100% in both cases, showing a null phytotoxic effect of the strains for this parameter. In fact, most of the strains produced an increase of the ISW parameter in relation to the control (distilled water), especially when they were applied at 2.0 $mg.mL^{-1}$. However, no significant differences could be established between the two applied concentrations.

3.2 Antagonistic cyanobacteria against the *in vitro* growth of *Pythium ultimum*

In this work, the entire collection of cyanobacterial extracts was analyzed to study its ability to inhibit the *in vitro* mycelial growth of the phytopathogenic oomycete *Pythium ultimum*, a causative agent of *damping-off* in cucumber seedbeds. A first set of tests was performed using the previously described dual culture protocol (see section 2.3.1.).

Approximately one third of all the cyanobacterial extracts, suggested some capability to delay the growth of the phytopathogenic oomycete (Fig. 2), although four of them induced small inhibition zones (% Inhibition < 14).–However, contrary to the aforementioned, five cyanobacterial extracts exhibited a remarkable inhibitory effect against PU growth, which varied from 40 to 50% inhibition (Fig. 2). The maximum inhibition recorded in this work corresponded to the strain SAB-M465, which managed to reduce the mycelial growth of PU more than 50%. For this reason, SAB-M465 was selected as the most potent antagonist strain to be further evaluated in detached leaf and *in planta* bioassays against PU (see sections 3.3. and 3.4.).

Figure 2. Inhibition Index (I%) derived from the application of sonicated extracts of cyanobacteria against the in vitro growth of Pythium ultimun. The figure shows the



mean of 4 replicates for each measure of the Inhibition Index. Vertical bars represent standard error values.

Thus, taking into account the three parameters studied *in vitro* with the complete collection of cyanobacteria (GI, ISW and I), a cluster analysis was carried out to classify all the strains studied into affinity groups (Fig. 3). Since the scale on which the different data sets were measured was the same (%), the distances calculated in the dendrogram were comparable. After analysis, five categories were observed, which helped to establish interesting relationships among the 31 strains analyzed. In fact, there was an exact coincidence in the cluster formed by the 5 antagonistic strains considered most significant against PU (Fig. 2). So, SAB-M134, SAB-M150, SAB-M465, SAB-M683 and SAB-B866 were categorized as a single group (“Remarkable antagonism” and “Null phytotoxicity”). Likewise, the 4 strains previously described with slight suppressive capacity (Fig. 2)

appeared grouped, most of them, SAB-B912, SAB-B974 and SAB-B1267, in a cluster categorized as “Non remarkable antagonism”, but “Remarkable phytostimulaton”, while the strain with the smallest antagonistic effect (SAB-B1572), was grouped in a cluster with the strains showing “Null antagonism” and “Mild phytostimulation”. The remaining two clusters corresponded to strains classified radically opposite from each other with respect to their level of phytotoxicity. Those strains showing “High phytotoxicity” were placed to the left of the dendrogram, while those strains with “Null phytotoxicity” were located to the right of the dendrogram (Fig. 3). Nonetheless, both groups agreed on their lack of antagonistic ability against the phytopathogenic oomycete. These results (Fig. 3) showed a potential relationship between the ability to combat plant pathogens and phytotoxicity (or lack of it).

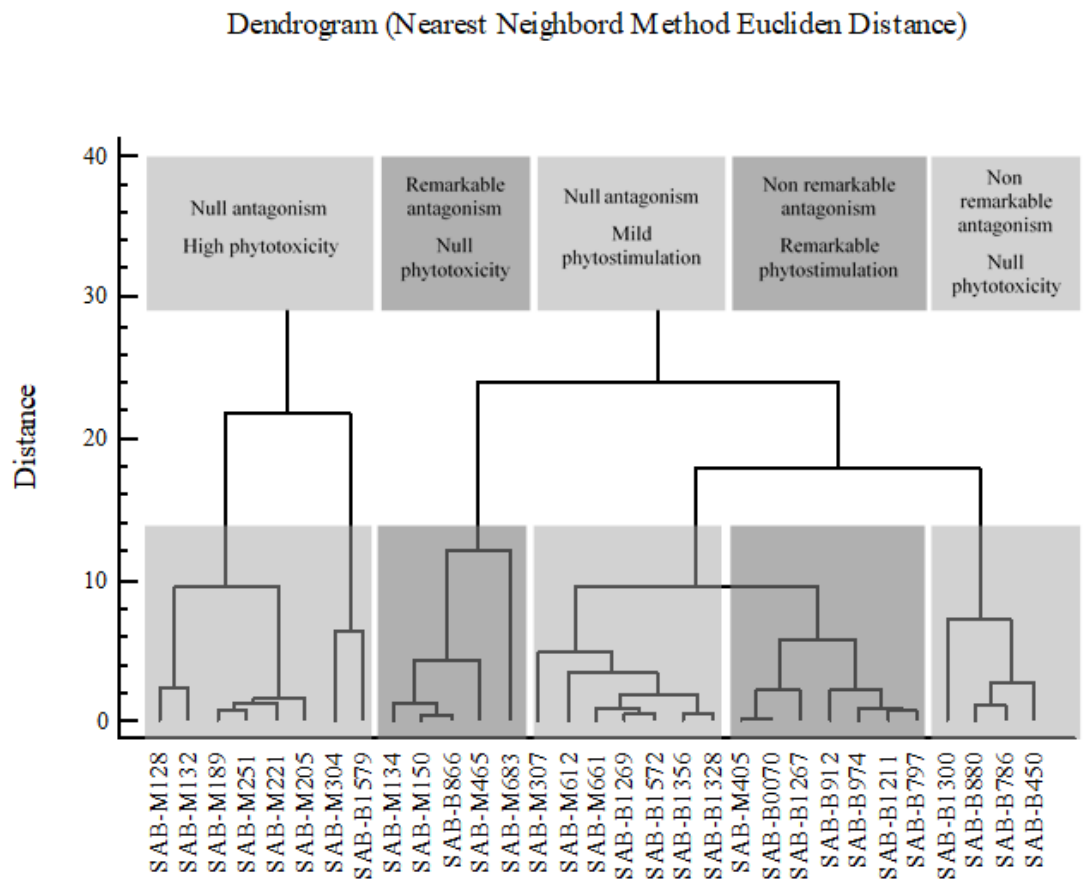


Figure 3. Dendrogram of the euclidean distance by the nearest neighbor method: clustering in vitro variables: Germination Index (GI%), Increase of Seedling Weight (ISW%) and Inhibition Index (I%).

3.3 Detached-leaf bioassay for evaluating resistance to *Pythium ultimum* after treatment with cyanobacterial extracts

As mentioned in the previous section (3.2.), the mechanisms to combat plant diseases involve both direct inhibition of the pathogen and activation of plant defense systems (Arora *et al*., 2013). Taking both premises into account, the strain SAB-M465 was selected as the most effective biopesticidal strain, while SAB-B912 was the best biostimulant strain on the basis of results previously described (sections 3.1. and 3.2.). Accordingly, Figure 4 shows the results derived from the detached-leaf bioassay for

evaluating resistance to PU after treatment with both cyanobacterial extracts.

The main objective of this experiment was to develop a rapid and effective bioassay to evaluate the potential of the two selected cyanobacteria to inhibit or control PU growth (Fig. 4). For this, freshly cut cucumber leaves were used, so that they remained living and turgid until the appearance of possible symptoms.

Figure 4 shows the results derived from the bioassay conducted on cucumber detached leaves infected with PU and previously treated with the cyanobacterial extracts SAB-MAC465 and SAB-B912. The bioassay was monitored at 2, 3 and 6 days from the start of the experiment. Both treatments were intended to evaluate the preventive effect of the selected strains against the development of a leaf injury by the pathogenic oomycete. In parallel, negative controls were prepared from distilled water and both cyanobacterial extracts (without PU). As expected, no signs of infection were observed at 48 h, 72 h and six days after inoculation in negative controls.

The effects of inoculating the detached cucumber leaves with the pathogen resulted in a clearly defined decay that rapidly spread to the entire leaf within a few days. In fact, 48 h after inoculation with PU, the lesion reached approximately 51 mm diameter on the inoculated control leaf (Fig. 4). At this sampling time, the development of necrotic damage was superior in the control with respect to both pretreatments. It should be noted that in the case of strain SAB-M465, the presence of the cyanobacteria extract inhibited the growth of PU more

significantly. The foliar lesion only grew about 36 mm diameter (SAB-M465) while it reached 48 mm diameter after the treatment with the strain SAB-B912. Both values supposed an inhibition rate around 30 and 10 %, respectively.

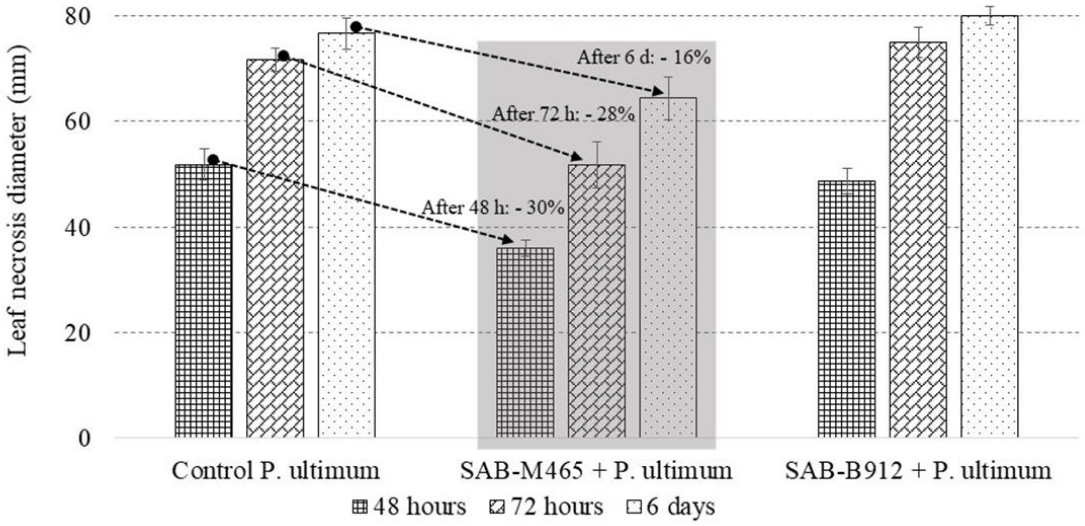


Figure 4. Detached-leaf bioassay showing the lesion diameter (mm) caused by Pythium ultimum on cucumber leaves pretreated with sonicated cyanobacterial extracts. Lesion diameter evolution was recorded at 2, 3 and 6 days after the beginning of the bioassay. Five replicates were measure for each dual culture. The figure shows the mean of 5 replicates for each measure of the diameter lesion. Vertical bars represent standard error values.

After 72 h, the diameter of the foliar lesions increased considerably in all the cases. In fact, a foliar area higher than 70 mm diameter was affected by PU in the control leaves exclusively inoculated with PU. Similar results were observed in the leaves previously inoculated with strain SAB-B912. Nevertheless, the foliar lesion in the leaves pretreated by SAB-M465 were notably smaller than those regarding control samples. In this case, the

diameter of the foliar lesions was around 50 mm, which meant an inhibition rate close to 30% with respect to the control.

Finally, after 6 days of incubation, both cucumber leaves used as control and those subjected to pretreatment with SAB-B912, presented similar results to those obtained at 72 h, reaching foliar lesions almost 80 mm in diameter. This fact could mean that SAB-B912 did not strictly inhibit the foliar necrotic damage, but it occurred more slowly at the beginning of the bioassay (48 h). Leaf injury caused by PU also continued progressing even in those leaves pretreated with SAB-M465. However, even after 6 days, an inhibition rate against the pathogenic oomycete was observed above 15%, in relation with that detected in control samples.

Summarizing, the development of foliar lesions caused by the oomycete *P. ultimum* was diminished (*in vitro*) in the presence of the extract SAB-M465 at different sampling times. This makes the SAB-M465 cyanobacterium a powerful Biological Control Agent (BCA), which was later applied *in vivo*. On the other hand, the discreet effect of strain SAB-B912 during the earliest phase of the detached leaf bioassay was decisive in deciding to continue working with this strain in subsequent bioassays carried out *in planta*.

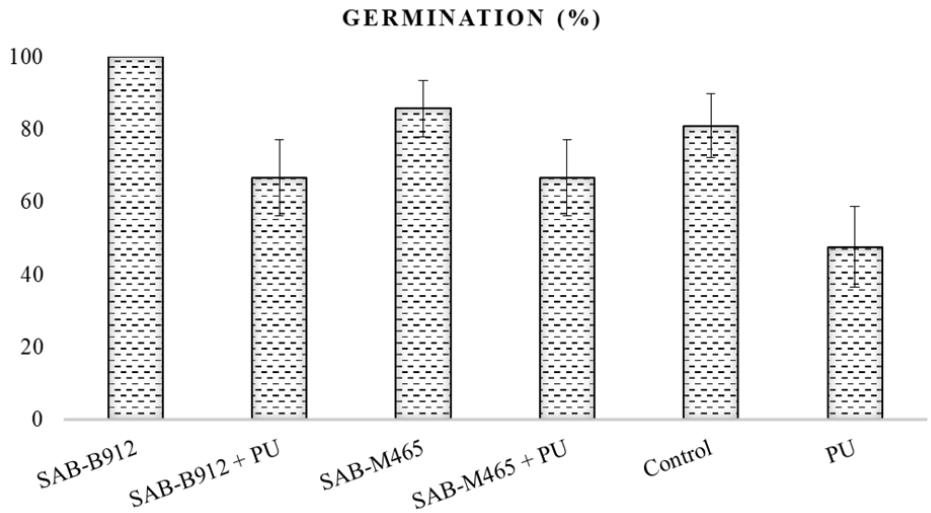
3.4 Early phytostimulation and plant protection against *damping-off* caused by *Pythium ultimum* by seed *biopriming* with cyanobacterial extracts

In order to examine the *in vivo* potential as plant growth promoters, microbial extracts from the strains SAB-B912 (eminently biostimulant) and SAB-M465 (eminently biopesticide) were applied on cucumber seeds (cultivar Ashley) susceptible to *damping-off.*

Figure 5 shows the results relative to Germination (%) of the bioimmersed cucumber seeds after 10 days from sowing in seed trays composed of 77 seedling cavities each. The determination of this parameter could help to evaluate the *damping-off* effect after PU inoculation of the different experimental blocks.

Figure 5. Germination percentage (with or without pathogen pressure) of cucumber seeds bioprimed with sonicated extracts from Tolypothrix sp. SAB-M465 and Anabaena sp. SAB-B912. Control: non bioprimed seeds which had been sown into non infected substrate; PU: non bioprimed seeds which had been sown into infected substrate. Germination % was calculated from bioassays carried out with 30 replicates. Vertical bars represent standard error values.

As observed in Figure 5, seed blocks not subjected to the



infectious pressure of PU, registered higher germination percentages than those sown in soil infected with PU (about 50%). It should be noted that the seed blocks subjected to pre-

treatment with cyanobacterial extracts exhibited germination percentages even higher than control (about 80%), reaching values near of 90 and 100% in the case of SAB-M465 and SAB-B912, respectively. Once again, SAB-B912 was able to promote the seed emergence stage as it was demonstrated in sections 3.1. Beyond what is described above, when pretreated cucumber seeds were sown in soil previously inoculated with PU, a decrease in the percentage of germination was generally observed, but always lower than detected from the PU infected control block. Thereby, data support a relevant cushioning effect on seeds treated with both cyanobacterial extracts, since the germination percentage (near 70%) exceeded the germination percentage registered from the seed block sown in soil infected with PU without cyanobacterial pretreatment (less than 50%). In view of the results observed in Figure 5, the treatment of cucumber seeds with extracts of cyanobacteria resulted in a significant reduction in *damping-off* (about 36%) in relation to what was observed in the untreated control infected with PU (Fig. 5).

In addition, once the sprouted seeds reached the seedling stage, different parameters associated with plant growth were evaluated to achieve a better understanding of the interaction among each cyanobacterial extract, cucumber seedling and PU, in the case of blocks previously infected with the pathogen oomycete (Fig. 6).

In view of the results observed in Figure 6, the phytostimulatory effect of the applied cyanobacterial extracts in relation to the untreated control block was evident (Fig. 6 a, b, c, d). Such effect

was more notable in the case of the extract obtained from the strain SAB-B912, thanks to which the stem and root length of the pretreated plant blocks increased as consequently did, plant total length (Fig. 6 a, b, c). Biostimulant effect was even more significant when root/stem ratio was calculated. Thus, both pretreatments (SAB-M465 and SAB-B912) showed a significant increasing on this parameter regarding to that detected in control plants (Fig. 6d).

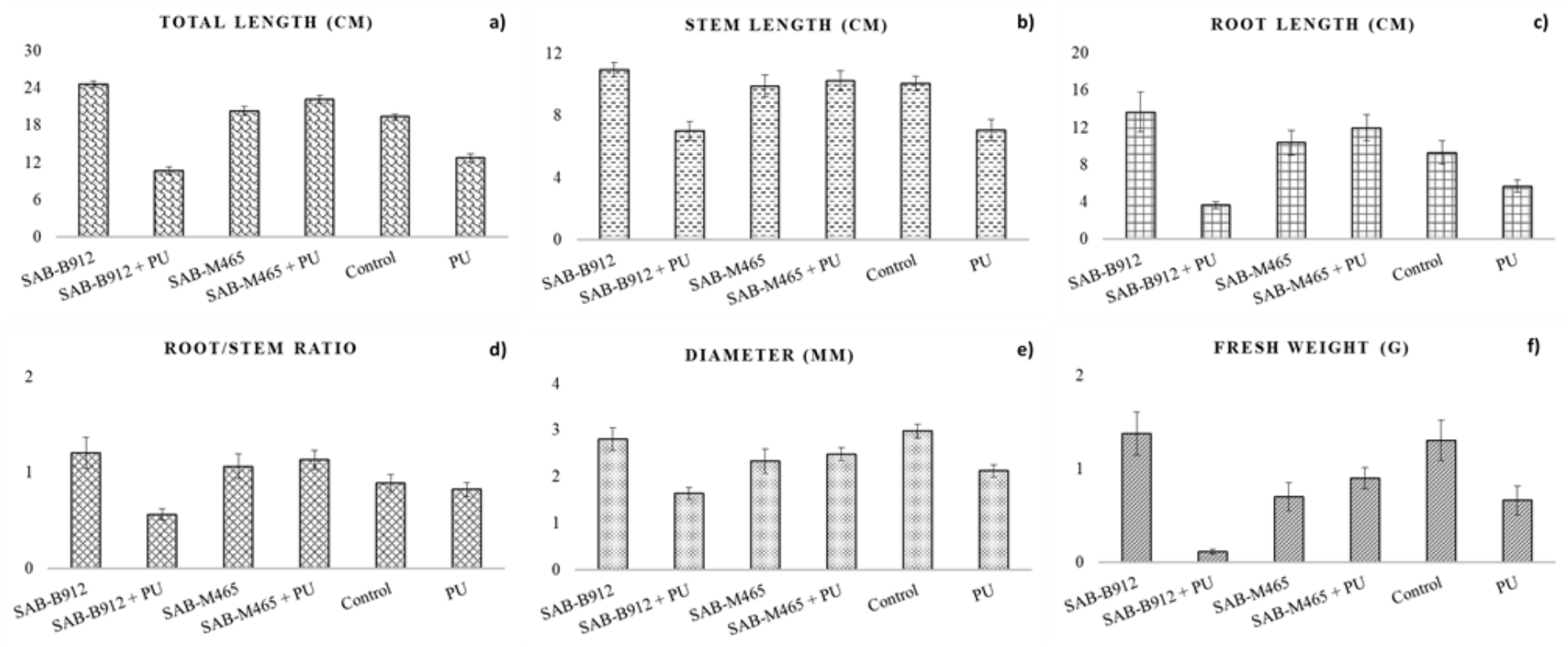


Figure 6. Measurement of plant growth parameters from cucumber seedlings previously bioprimed with sonicated extracts from *Tolypothrix* sp. SAB-M465 and *Anabaena* sp. SAB-B912. a-f) Total length (cm), stem length (cm), root length (cm), root/stem ratio, stem diameter (mm) and fresh weight (g) are represented. Mean of 30 replicates was used in each case. Vertical bars represent standard error values.

Therefore, plant promoting impact was remarkable when plant size (stem and root length) was considered but such effect was not observed in terms of thickness and fresh weight of the seedlings (Fig. 6e, f). Regarding these parameters, the plants treated with SAB-B912 were not different from the controls (without pretreatment), while the block treated with SAB-M465 were even thinner and showed a lower fresh weight than that shown by the control plants (Fig. 6e, f).

As indicated at the beginning of this section, the *biopriming*-based bioassay had two objectives: (i) to determine the capacity of cyanobacterial extracts to promote plant development during the early stages of plant growth and (ii) to verify the cushioning effect of both strains on PU-infected substrates. In this sense, the observation of the suppressive effect of *damping-off* in *bioprimed* seeds with the SAB-M465 extract was surprising. Stem and root length related data were greater in the case of blocks pretreated with SAB-M465, thus controlling the negative effect of the pathogen oomycete in relation to the size of the plant (Fig. 6a, b,c,d). Such results improved even to those observed in plants not infected with PU. Additionally, the above cited cushioning effect was also detected when stem diameter and plant fresh weight were considered (Fig. 6e, f). Contrary to what was indicated in the case of SAB-M465, plants pretreated with SAB-B912 extracts could not control the pressure of the pathogen present in the infected substrate, giving rise to small plants in which the root system was clearly affected (Fig. 6a-f).

4. Discussion

Numerous investigations support the preventive effect that various biological control agents exert on the development of *damping-off*, avoiding the colonization of plant tissues (seed and roots) by pathogens (Georgakopoulos *et al*., 2002; Elshahawy and El-Mohamedy, 2019). However, prevention through seed pretreatment has traditionally been carried out through the use of bacteria and fungi as control agents (Schoebitz *et al*., 2013; Mahmood *et al*., 2016; O'Callaghan, 2016), while the participation of cyanobacteria in this type of protocol is still preliminary.

Numerous investigations have confirmed the positive effect of the application of cyanobacterial extracts at low concentration (Dmytryk *et al*., 2014; Aghofack-Nguemezi *et al*., 2015; Godlewska *et al*., 2019). Godlewska *et al*. (2019) demonstrated the stimulating effect of extracts of *Spirulina platensis* on seed germination. Although all concentrations positively influenced radicle length, the data was less surprising at higher extract concentrations.

According to Zucconi *et al*. (1981), GI above 70-80% indicates absence of phytotoxicity, while around or above 100% are indicative values for plant-growth promoting. GI and ISW describe the phytotoxicity or phytostimulant phenomenon from a point of view that integrates both relative germination percentage and relative root growth, with respect to its length and weight, respectively (Zucconi *et al*., 1981; Emino and Warman, 2004). On the basis of the results here described, the combination of both parameters is a more sensitive indicator of phytotoxicity than the simple calculation of germination percentage. This is

observed, despite, or precisely due to the contrast of results found when all the data collected were available. Thereby, while GI verified that the elongation of the radicle is lower as the concentration of the cyanobacterial extracts increased, ISW revealed to be less sensitive to that factor. In this sense, several authors pointed out the great differences observed during the growth of the roots in seeds exposed to the presence of moderately phytotoxic metabolites, but somehow influenced the development of the radicle (Emino and Warman, 2004; Varnero *et al*., 2007). In the present work, it has been shown that all cyanobacterial extracts influenced (in general) positively the fresh weight of watercress after germination what confirms literature data (Mógor *et al*., 2018; Godlewska *et al*., 2019).

Several species of cyanobacteria, known primarily for their role as nitrogen supplements in fields, are also currently considered as potential biocontrol agents. Many of them exhibit antagonistic effect against plant pathogens such as bacteria, fungi and nematodes, mainly as a result of the production of a variety of biologically active compounds (Prasanna *et al*., 2008; Prasanna *et al*., 2010; Gupta *et al*., 2013; Natarajan *et al*., 2013). The biocontrol efficacy of these agents is most likely related to numerous mechanisms of action that include production of antibiotics (Prasanna *et al*., 2009) and other antimicrobial substances such as hydrolytic enzymes, biocidal compounds such as benzoic and majusculoic acids, or bioactive molecules like siderophores or phytohormones (Natarajan *et al*., 2012; Singh, 2014).

Several cyanobacterial compounds are involved in the biological control of diseases that affect vegetable crops. This is the case of several antibiotics that contain chlorine in the case of *Scytonema* spp., majusculamide in the case of *Anabaena laxa* or benzoic acid from *Calothrix* spp. (Natarajan *et al*., 2012; Singh, 2014). In many cases, they improve the immunity of plants by causing the activity of plant defense enzymes (Prasanna *et al*., 2008). Thus, chitosanase and endoglucanase-type enzymes in various strains of *Anabaena* spp. and *Calothrix* spp. seem to show some similarity with other enzymes of fungal and bacterial origin (Gupta *et al*., 2013; Natarajan *et al*., 2013).

Previous studies, related to the antagonistic effect against different phytopathogens associated with *damping-off* disease, yielded up low susceptibility of PU after being confronted with strains considered as biocontrol agents (Jurado *et al*., 2019). Sánchez-San Fulgencio *et al*. (2018) reported the insignificant inhibitory effect of some compost microorganisms against PU, compared to the effect observed against other fungi or oomycetes responsible for *damping-off*. However, significant antagonistic activity against vascular pathogens has been attributed to cyanobacterial extracts. Some strains identified as *Nostoc muscorum* have been shown to be effective against the growth of *Candida albicans* and *Sclerotinia sclerotiorum*, thanks to the production of bioactive compounds (Stratmann *et al*., 1994; Zulpa *et al*., 2003). Furthermore, some strains of *Nostoc* spp. have been considered capable of inhibiting fungi *damping-off* producers, such as *Fusarium oxysporum, Penicillium expansum, Rhizoctonia solani, Sclerotinia sclerotiorum, Verticillium*

alboatrum and *Phytophthora cinnamomi*, thanks to the action of microbial methanolic extracts (Biondi *et al*., 2004). In addition to *Nostoc* spp., other cyanobacteria such as *Anabaena* spp. and *Scytonema* spp. showed antifungal activity against *Fusarium oxysporum* fsp. *lycopersici, Pythium* spp. and *Rhizoctonia solani* (Kim and Kim, 2008; Yadav *et al*., 2018). Manjunath *et al*. (2010) evaluated biocontrol potential of filtered extracts of *Calothrix* spp., finding them effective for the control of *damping-off* and other plant diseases. Likewise, Dukare *et al*. (2011) revealed the utility of inoculating compost with the cyanobacterium *Anabaena oscillarioides* to provoke resistance against phytopathogenic fungi that cause *damping-off* in tomato plants. More in detail, some authors revealed the high efficiency of the cyanobacterial culture filtrates, such as those from *Spirulina platensis* and *Nostoc muscorum*, in suppressing the mycelial growth diameter of several phytopathogenic fungi. This antifungal activity was attributed to the presence of bioactive substances that are, phenolic compounds, proteolytic enzymes and high levels of indol-acetic acid (IAA) (Tantawy, 2011; Prasanna *et al.,* 2013).

Detached leaf bioassays have been previously used as a quick and easy method to assess the virulence of different fungal pathogens causing foliar plant diseases (Pettitt *et al*., 2011; Cowley *et al*., 2012) and, therefore, they can be used to detect potential Biological Control Agents (BCAs) (Saxena and Pandey, 2002). This methodology has proven to be very useful for evaluating the antagonistic capacity of various BCAs against molds and oomycetes, despite the fact that the rapid and invasive mycelial growth of both fungal groups could interfere *a priori* with

the optimal development of this type of bioassay (Park *et al*., 2008; Guo *et al*., 2015, 2016).

Based on previous work, it is known that several strains of *Anabaena* and *Calothrix* exerted activity against species of *Pythium*, *Fusarium* and *Rhizoctonia* (Prasanna *et al*. 2008; Manjunath *et al*. 2010). Among all the compounds synthetized by cyanobacteria, chitosanase homologues, endoglucanase and benzoic acid were detected, and their presence was correlated to the activity against phytopathogenic fungi and oomycetes (Gupta *et al*. 2010, 2011; Prasanna *et al*. 2010). Chitosanase enzymes selectively decompose chitosan and chitin by hydrolysis of the β-(1,4)-glycosidic bonds that link N-acetyl glucosamine residues of chitin. This bioactivity could be partly responsible for the inhibition of PU growth in the detached-leaf bioassay.

Although the use of bioassays of detached parts of plants has been widely used in plant pathology, some authors have reported that there could be limitations derived from the possible deterioration of the plant tissue before the bioassay be successfully completed (Pettitt *et al*., 2011). However, in this work, this drawback was not observed, given the speed with which the leaf lesion developed in the cucumber detached leaf due to PU inoculation.

Attia *et al*. (2016) stood out that controlling or inhibiting the development of diseases due to plant pathogens was involved in direct suppression, through the secretion of allelochemicals or secondary metabolites, or indirectly, through the induction of systemic resistance, by producing phytohormone like

substances, or eliciting the antioxidant and pathogenesis related machinery of the plant (activity of β-(1,3)-endoglucanase, chitinase, catalase, peroxidase, polyphenol oxidase or phenylalanine ammonia lyase) (Gayathri *et al*., 2015). All this, although useful to enhance protection against plant pathogens, would not always lead to direct benefits for plant growth and development. However, in view of the results obtained, one might suspect that the seed treatment with cyanobacterial extracts before sowing can play an important role in increasing crop productivity, since it improves seed quality and germination capacity (Singh *et al*., 2015). The effect of such treatments in the early stages of plant development could be related to better nutrient absorption, the presence of protective substances and growth regulators. In the present study, cucumber seeds were treated with cyanobacterial extracts that could serve as a source of new biochemically active natural compounds. A clear example of this is described in the work of Mógor *et al*. (2018), where the increase of the fresh biomass of cucumber and lettuce was attributed to the cytokinin-like activity of Spirulina extracts.

Traditionally, other different methods are known to apply beneficial microorganisms as a protection measure against phytopathogenic agents. Some examples are foliar application by spraying, direct inoculation in the rhizospheric environment or as part of some type of organic amendment (compost) (Mahmood *et al*., 2016). However, the strategy applied in this work, *biopriming*, consists of immersing the seeds in a microbial extract for a set time, in order to achieve a lasting and effective primer that ensures plant physiological changes inside the seed

just before the emergence of the radicle and plumule. This technique has been shown to improve germination and seedling status, ensuring later the proper growth and yield of crops (Anitha *et al*., 2013).

In this work, the cyanobacterial extracts were applied in a semi-solid matrix made of sodium alginate to ease the colonization on the seed coat. Multiple possibilities have been highlighted regarding the use of polysaccharides such as sodium alginate, carboxymethyl cellulose or xanthan gum, among others (Angelopoulou *et al*., 2014; O'Callaghan, 2016). In this sense, alginate has been considered one of the most widely used options in semi-solid microbial formulations showing an improved ability of microorganisms to colonize plant roots without reducing the cell viability (Schoebitz *et al*., 2013).

Cyanobacterial extracts could act not only as antifungal agents directly, but also improving the antagonistic capacity of other biological control agents in the rhizospheric environment (fungi, bacteria or yeasts), thanks to the production of bioactive substances that also stimulate microbial activity (El-Mougy and Abdel-Kader, 2013). Therefore, the suppression of *damping-off* through the action of biocontrol agents occurs as a consequence of the interactions occurring among soilborne pathogen, plant and beneficial microbial community (Singh and Sachan, 2013). Though this fact could affect the protective capabilities of the biological control agent and modify the results observed *in vitro* (Errakhi *et al*., 2007), an effectiveness of up to 35% in the protection of cucumber seedlings against PU has been demonstrated in this work by the preventive protocol described

as *biopriming*. Such results are quite promising in relation to the design of biological control strategies based on the use of cyanobacterial extracts.

5. Conclusions

The strain SAB-B465 has positioned itself as an excellent control agent against the *damping-off* caused by *P. ultimum* in cucumber cultures. This effect has been shown in early stages of plant development, such as pre and post- emergence, thanks to a promising *biopriming*-based protocol intended to be a preventive seed treatment. Parallel to the cushioning effect of the disease, this strain showed a slight stimulating effect on root development, which could be related to the strengthening of the plant to counteract *P. ultimum* damage. Contrary to what was observed in the case of SAB-M465, SAB-B912 showed a clear growth promoting effect in the early stages of plant development, but it failed to decrease the damage caused to cucumber seedlings grown on PU-infected substrate. This fact coincided in part with what observed in *in vitro* tests.

In view of the results above described, protocols applied in this work aimed at the preliminary characterization of the cyanobacterial collection were very useful for predicting phytotoxic, phytostimulating and biopesticidal capacities. Likewise, the use of the *biopriming* strategy in cucumber seeds was successful as a preventive treatment of *damping-off* caused by *P. ultimum*, as well as to apply extracts derived from cyanobacteria capable of promoting plant development in pre- and post-emergence phases. Therefore, seed *biopriming*

strategy based on cyanobacterial extracts could be considered a promising environmentally sustainable and non-phytotoxic alternative, aimed at the preventive treatment of seeds against fungi and phytopathogenic oomycetes producing *damping-off* in seedbeds.

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7. Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version.

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The following are the Supplementary data to this article:

Supplementary Table S1

Table S1. Collection of cyanobacteria studied in this work: code, origin and scientific name.

Code	Isolated from	Scientific name
SAB-B0912	Seawater	*Anabaena sp.*
SAB-B1572	Seawater	
SAB-M307	Freshwater	
SAB-B0797	Seawater	*Calothrix sp.*
SAB-M405	Freshwater	
SAB-B1269	Seawater	*Cyanobacteria sp.*
SAB-B0866	Seawater	*Dolichospermum sp.*
SAB-B0070	Seawater	*Gloeocapsa sp.*
SAB-B1211	Seawater	*Leptolyngbya sp.*
SAB-B1267	Seawater	
SAB-B0450	Seawater	
SAB-B1328	Seawater	*Lyngbya sp.*
SAB-B1356	Seawater	*Nodularia sp.*
SAB-B1300	Seawater	*Nostoc sp.*
SAB-B0786	Seawater	
SAB-M132	Freshwater	
SAB-M150	Freshwater	
SAB-M189	Freshwater	
SAB-M251	Freshwater	
SAB-M612	Freshwater	
SAB-M661	Freshwater	
SAB-M683	Freshwater	
SAB-B0880	Seawater	*Phormidium sp.*
SAB-B1579	Seawater	*Synechococcus sp.*
SAB-M205	Freshwater	*Tolypothrix sp.*
SAB-M465	Freshwater	
SAB-B0974	Seawater	*Trichormus sp*
SAB-M304	Freshwater	
SAB-M128	Freshwater	

SAB-M134	Freshwater
SAB-M221	Freshwater

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