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Optimization of the production of herbicidal toxins by the fungus *Ascochyta caulina*

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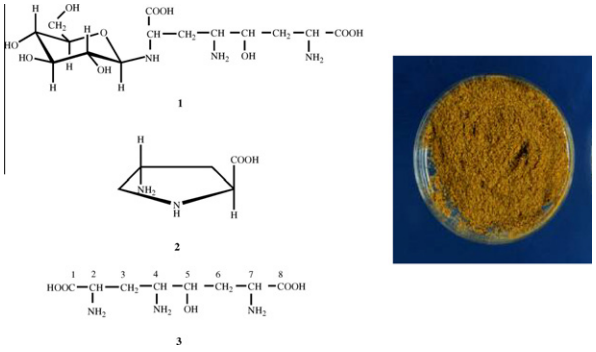
HIGHLIGHTS

- Production of natural metabolites with herbicidal properties by a liquid shaken fungal culture.
- Optimization of fungal growth and metabolite production.
- Minimization of costs of production.
- Set up of the conditions for production of the natural herbicide through a fermentation unit.

GRAPHICAL ABSTRACT

Ascaulitoxin, trans-aminoproline and ascaulitoxin aglycone (1, 2 and 3, respectively, left), three fungal metabolites with herbicidal properties, can be produced at a pre-industrial level by growing the fungus *Ascochyta caulina* in a fermentation unit and effectively purified as a mixture (right).



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ABSTRACT

Safer and more environmentally friendly methods, including the use of natural substances, would be favorably considered for weed management, particularly in those habitats where the use of chemicals is restricted or banned. *Ascochyta caulina*, a proposed mycoherbicide for biocontrol of the weed *Chenopodium album*, produces in liquid culture three main metabolites proposed as possible natural herbicides. Recently a research project, named ECO-VIA, initiated a series of studies aimed at developing the technologies to obtain a natural herbicide based on these bioactive metabolites. Particular attention was given to: maximizing toxin production; lowering the production costs; scaling up the production in fermentation systems; setting up a large scale purification method and identifying fast and inexpensive chemical methods to quantify toxins yields. The fungus proved to grow well and to produce up to 230 mg of toxins l^{-1} culture when grown for 5–10 days in shaken conditions, provided the initial inoculum was at least 10^5–10^6 conidia ml^{-1} of culture. Toxin production was improved by the addition of yeast extract to the medium. Given suitable inoculum and growth conditions, a stirred fermenter could be suitable for mass production of toxin.

1. Introduction

Weed management is particularly difficult in areas having public, residential or industrial uses, such as recreational or archaeological parks, pavements, roadsides, or railroads, where the use of chemical herbicides is severely restricted or mostly banned, and other control methods are limited or costly. Safer and more environmentally friendly methods, including the use of natural substances would be considered favorably by the public, politicians and the scientific community although very few practical applications have been implemented yet. *Ascochyta caulina* (P. Karst.) Aa & Kesteren is the

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fungal causal agent of a disease of *Chenopodium album L.*, a worldwide noxious weed (Holm et al., 1977). This pathogen has been studied and proposed as a possible agent for the biological control of that weed (Kempenaar, 1995a; Vurro et al., 2001). Previous studies have led to the production, purification, and chemical and biological characterization of three main toxins from liquid culture filtrates of the fungus, namely ascaulitoxin, ascaulitoxin aglicone and trans-4-amino proline (Evidente et al., 1998, 2000, 2001). Their use in mixture as a natural and environmentally friendly herbicide was proposed on the basis of a series of relevant biological and technological properties (i.e., small molecular size, broad spectrum phytotoxicity to different plant species, lack of antibiotic and zootoxic activities, and full solubility in water). One of the main problems when studying bioactive metabolites produced by microbial organisms is the relatively low amount they produce *in vitro*. Indeed, in the conditions initially developed for the growth of the fungus (i.e., growth in a mineral defined liquid medium in static conditions for four weeks) ascaulitoxin, its aglycone and trans-4-aminoproline were produced on average at around 80, 240, and 30 mgl^{-1} of culture, respectively (Evidente et al., 2001). Those amounts could be sufficient on the lab scale to study the purification procedures, for their chemical characterization, for the development of their analytical methods, or for evaluating their herbicidal or other biological properties. However, the possibility to produce them by a simple, inexpensive and enantioselective synthesis is seriously hindered by the presence, for example in the ascaultixin aglycone, of some chiral variants, whose relative stereochemistry has been identified (Bassarello et al., 2001). When planning greenhouse or field experiments larger amounts of the compounds would be necessary. Furthermore, in view of scaling up microbe growth and metabolite production at a pre-industrial level, it becomes necessary to consider many other aspects that are not usually considered at the lab scale, i.e., to use liquid media and shaken conditions as is provided by a stirred fermentation system rather then a solid and still one; to evaluate the costs and the time of production, to maximize the toxin yields; to adopt easy inoculation and fermentation procedures; to develop fast and inexpensive methods of toxin analysis and quantification; and to employ reusable and environmentally friendly resins and solvents for their purification. Recently a research project named ECO-VIA, was approved with the support of the Regional Governorate of Lombardy within a specific programme for the development of the competitiveness of small and medium enterprises, made possible some progress in the development of technologies to obtain a natural herbicide based on the bioactive metabolites produced by *A. caulina*. This report is focused on the optimization of the cultural conditions of the filamentous fungus *A. caulina* in order to define the optimum conditions for the biosynthesis of the three toxins to be developed as natural herbicides in a pre-industrial system.

2. Materials and methods

2.1. Fungal strain

The strain of the fungus, *A. caulina*, was originally isolated from naturally diseased *C. album* plants (Evidente et al., 1998). It is stored in the ISPA mycological collection (ITEM 1058). The fungus, kept at 4° C, was routinely grown on potato-dextrose-agar (PDA) plates or slants as described by Evidente et al. (1998), and used fresh for the experiments.

2.2. Improvement of inoculum production

The standard method for the inoculum (conidia) production consisted of growing the fungus on potato dextrose agar (PDA) in Petri dishes for around two weeks at 25 °C under near UV lights. After this period of time the surface of the plates was covered by a dense mycelial mat containing many pycnidia. The conidia were harvested by pouring some milliliter of sterile distilled water on the plate surface, and then gently scraping it by using a spatula. The conidial suspension was finally collected in a sterile vial, the volume adjusted to 10 ml, and conidia counted by using a hemocytometer. For producing larger amounts of fungal inoculum, two other methods of conidial production were considered, beside the standard procedure.

2.2.1. Production of conidia on cellophane disks

The surface of petri dishes (containing PDA) was covered with a sterile cellophane disk (autoclaved at 115° C for 20 min) (Cellophane Disc Cell, PT). One hundred microliter of a conidial suspension obtained as described above was evenly poured on the disk. Plates were kept in a growth chamber under near UV light for 2 weeks. After growth, the plate surfaces were gently scraped, and the soft jelly obtained harvested using a sterile spatula, then placed in a sterile vial and dispersed with 10 ml of sterile distilled water. Conidia concentration was determined by using a hemocytometer.

2.2.2. Production of conidia on a wheat-bran based medium

A procedure previously described by Kempenaar (1995b) was adapted and used. Erlenmeyer flasks (500 ml) were filled with a mixed media composed of: 15 g spelt bran, 45 g quartz sand (50–70 mesh, Sigma) and 20 ml distilled water. Flasks were sterilized twice (121 °C for 30 min) on two successive days. Each flask was inoculated with a spore suspension (10 ml) containing a total of around 10^8 conidia prepared as described above, Flasks were then kept in a growth chamber, in the dark at 26 °C, up to one month, and periodically checked for conidia production. Conidia were counted by collecting 1 g of the medium at regular intervals, suspending it in distilled water and then counting the number of conidia with a hemocytometer.

2.2.3. Evaluation of conidial germination capability

In order to ascertain if inoculum preparation and media composition could negatively affect the capability and promptness of the conidia to germinate, different media were considered. Experiments were carried out by suspending conidia in: (1) distilled water; (2) the defined mineral medium (named M1-D – Pinkerton and Strobel, 1976) used as medium in the previous studies for *A. caulina* toxin production and characterization and used throughout these studies; (3) M1-D with commercial ammonium tartrate ($5\ g\ l^{-1}$); or, (4) M1-D with *C. album* leaf decoction, prepared (as reported in Section 3c). A conidial suspension (100 μl, approximately 10^3 conidia ml^{-1}) obtained as previously described, was placed in each well of a 3-well sterile glass slide and incubated in petri dishes in the dark at 25 °C. Conidia were observed every two hours, and germination was determined after 24 h and is expressed as percentage. A conidium was considered germinated if the germ tube length was at least equal to the width of the conidium. Approximately 100 conidia were counted for each well, with three replicates. The experiment was repeated twice.

2.3. Improvement of fungal growth and toxin production

2.3.1. Influence of inoculum quantity, shaking speed and growth time

Preliminary attempts, carried out to tentatively assess the capability of the fungus to be grown in shaken conditions instead of the standardized static condition, showed that the fungus did not grow well at rotation levels less than 50–60 rpm. As a result the following experiments were conducted. Flasks (250 ml) each containing 100 ml of the standard medium were inoculated with the spore suspensions prepared as above described and grown at 25 °C in

an orbiting shaker (CERTOMAT® IS Sartorius Stedim Biotech). Three different conidial concentrations were used: 10^6, 10^7, 10^8 conidia flask^{-1} at two different shaking speeds (80 or 120 rpm). Flasks were collected after 5 or 10 days, and three replications were prepared for each treatment. Each flask was filtered through Whatman No. 4 filter paper and the fresh and dry weights of the filtered mycelium was determined. The pH of the culture filtrates was measured and twenty-five milliliters of the culture filtrate were lyophilized and the concentration of the three toxins determined as described in Section 4. Data were subjected to the UNIANOVA analysis by using the PASW Statistics 18 package (SPSS Inc., Chicago, USA). The experiment was repeated twice.

2.3.2. Effect of additives and inoculation methods

The influence of surfactants was evaluated by adding Tween 80 (Sigma–Aldrich) or glycerol (Carlo Erba Reagenti, Milan) (both at 0.01% –v:v) to the standard medium prepared as described above. Flasks were inoculated with a total of 10^8 conidia flask^{-1} and grown at 120 rpm for 10 days. Fungal growth and toxin production was determined as described above and compared with those of the standard culture.

The standard inoculation procedure consists of preparing fresh conidial suspensions from actively grown plates prepared as above described and the use of 1 ml (containing 10^6 conidia) of that suspension to inoculate each flask. Two additional different inoculation procedures were used and compared to the standard procedure: (1) use of fragmented (chopped) mycelium; and (2) use of pre-germinated conidia. For the preparation of (1) a 1 ml conidial suspension with 10^6 conidia was placed in a flask containing PDB (potato dextrose broth – Difco™) and grown for 4 days at 25° C in the dark under shaken conditions (100 rpm). The mycelium was harvested by gravity filtration through sterile gauze, resuspended in 100 ml sterile M1-D medium and ground for 1 min by using a blender at high speed. Larger mycelium fragments were removed by filtration. Ten milliliter of the suspension were used to inoculate each flask containing the standard medium, with the addition of Tween 80 (0.1%). For the preparation of (2) the same spore suspension used to prepare (1) was kept under shaking conditions for one day to allow conidial germination. Conidia were harvested by centrifugation, resuspended in M1-D, and 10 ml were used to inoculate the standard M1-D medium with added Tween 80. Flasks were grown in a rotary shaker at 120 rpm and at 25 °C for 10 days. Cultures were evaluated as described below. Three replications were prepared for each type of inoculum, including the standard one, used for comparison, as control. The experiment was repeated twice. Data were subjected to the ANOVA statistic factorial analysis by using the PASW Statistics 18 package.

2.3.3. Effect of nutritional elements

In order to optimize fungal growth, maximize toxin production, and reduce the costs of the medium, a number of different nutritional elements were tested. In a preliminary experiment, the standard recipe of M1-D medium was modified by changing the initial pH, replacing sucrose with the same amount of different sugars, increasing or reducing the amount of ammonium tartrate, replacing the nitrogen source, or adding vitamins. The modified media used are listed in Table 4. Sugars were used at the same concentration as saccharose in the standard original recipe, as well as the nitrogen sources, unless otherwise specified. After 10 days, fungal mycelium was separated from the culture filtrate by vacuum filtration and the pH determined. Twenty-five milliliter of culture filtrate were collected from each sample, lyophilized and the total content of the three toxins determined as described below. In a second experiment, all of the media were prepared with the addition of Tween 80 (0.1%) and some of the compounds already used in the first experiment were used again. Moreover, the standard M1-D medium was prepared with the addition of a decoction of *C. album* leaves. The decoction was prepared by boiling *C. album* leaves in distilled water (2 or 10 g l^{-1}) for 30 min. Leaves where then removed by filtration and the filtrate was used to prepare the medium as already described. The modified media used are listed in Table 5. Cultures were grown for 10 days after inoculation. Some cultures were harvested 6 days after inoculation, as they started turning brown. Fresh and dry weights of the filtered mycelia, pH of the culture filtrates, and toxin content were determined as above described. Three replications were used for each treatment. The experiment was repeated twice. Data were subjected to the ANOVA statistic factorial analysis by using the PASW Statistics 18 package.

2.3.4. Use of hydrogel

Preliminary observations were carried out to evaluate the capability of the fungus to be grown in a solid–liquid fermentation using a hydrogel. A protocol used for formulating biological control agents was adapted (Connick, 1988). Briefly, sodium alginate (20 g l^{-1} – Sigma) was added to a conidial suspension (10^6 conidia ml^{-1}) prepared as described above. The alginate/spore suspension was stirred continuously while it was dripped through a syringe with a 1 mm diameter orifice into a sterile $CaCl_2$ solution (0.2 M, Carlo Erba Reagents, Milan) under continuous shaking conditions. After contact, the droplets were instantly transformed into small hydrogel spheres. Pellets were collected, washed several times with M1-D to remove $CaCl_2$ excess, and placed in 100 ml M1-D flasks with 0.1% of Tween 80. All procedures were carried out in aseptic conditions. Flasks with hydrogel spheres were kept in a rotary shaker under the same conditions as described before. Standard M1-D with added Tween 80 was used as a control. The experiment was repeated twice in triplicate. After 10 days the culture was filtered and the toxin content was determined as described above. Pellets were also placed in PDA plates to verify the fungus viability.

2.4. Fermentation scale-up

Attempts were made to assess the suitability of the fungus to be mass produced in a stirred fermentation system and to test its ability to produce the toxins in those conditions. For this, a fermenter (BIOSTAT C 30 – Sartorius Stedim Biotech) was used, and three runs were arranged, each time slightly modifying the protocol used in order to progressively fine tune the system.

In the first fermenter run, 15 l of standard M1-D was prepared. The sterilized medium, added directly into the fermentation system, was inoculated with 500 ml of the conidial suspension containing approximately 10^6 ml^{-1}. This inoculum was obtained from 40 plates prepared on PDA as described above. Fermentation was carried out at 40 rpm, at 25 °C, and with an air flux between 0.45 and 2 l per minute, for 14 days. Starting on the 3rd day after inoculation, 10 ml of the culture medium were collected every 3 days, and pH and toxin content of the filtrates were determined.

In the second run the fermenter was used at full capacity (30 l). In this test M1-D medium was used with Tween 80 (0.1%). The medium was inoculated with 400 ml of conidial suspension (4×10^6 conidia/ml^{-1}) prepared by using the wheat-bran based medium (as above described). The cultures were grown at 80 rpm and an air flux of 1–1.5 lmin^{-1} for 15 days.

The third run was carried out as the second one although a difference was that every 2 or 3 days the rotation speed was increased to around 200 rpm for two or three minutes, and then restored to 40 rpm to reduce adhesion of the mycelia lumps to the fermenter walls and blades.

2.5. Purification, analysis and quantification of the toxin content in the culture filtrates

The culture filtrates, obtained from cultures grown under the different conditions reported above, were purified and analyzed according to a method recently described by Avolio et al. (2011). Briefly, the lyophilized filtrates (corresponding to 25 ml) were purified by Ion Exchange-Solid Phase Extraction using a Visiprep Vacuum Manifold (Supelco, Bellefonte, PA, USA), by dissolving them in 2 ml of 1 M formic acid and loading them onto the column (SPE, 6 ml). Toxins were eluted by ultrapure H_2O (3 ml) followed by 1 M NH_4OH (5 ml). The basic eluate was fluxed by a N_2 stream. The toxins were analyzed by HPLC (Shimadzu LC-10 AD-VP) using a column POLYSEP-GFC-P-3000 (Phenomenex 300 × 7.80 nm). Ultrapure H_2O was used as the mobile phase. The flow-rate was 1 ml min^{-1} and the UV–VIS detector was positioned at 200 nm. Samples were injected using a 20 μl loop, and eluants were monitored for 20 min. Toxin quantification was carried out by comparison of the analytical data with the calibration curves of the three toxins obtained by measuring solutions containing the pure toxins in the range of 2–200, 4–315, and 3–260 μg for ascaulitoxin, its aglycone and *trans*-4-aminoproline, respectively as described in details by Avolio et al. (2011).

3. Results

3.1. Improvement of inoculum production

3.1.1. Production of conidia on cellophane disks or wheat-bran based medium

When grown using cellophane disks, isolate AC1 produced on average on each plate $5.54 \pm 1.5 \times 10^7$ conidia, compared to $1.82 \pm 0.34 \times 10^7$ conidia obtainable from the standard method on PDA plates. After one week of growth on cellophane disks the fungus produced a large number of pycnidia, and conversely a limited amount of mycelium, resulting in a more abundant amount of collectable conidia. Grown on wheat-bran based medium, the fungus produced on average $2.14 \pm 0.26 \times 10^8$ conidia g^{-1} of fresh material.

3.1.2. Evaluation of conidial germination capability

The conidial germination rate was calculated as a percentage of the total conidia counted. After two hours, approximately 30% of the conidia suspended in the medium added with 10% *C. album* leaf extract had already germinated. After six hours, all the conidial suspensions, with the exception of that prepared in distilled water, showed a germination percentage close to 100%. At 24 h the germination percentage of the conidia suspended in water control reached around 40%, still significantly lower than the other suspensions.

3.2. Improvement of fungal growth and toxin production

3.2.1. Influence of inoculum quantity, shaking speed and duration on the growth

When grown at 80 rpm the fungus performed better with the intermediate amount of inoculum, raising a total of more than 220 mg of toxin mixture l^{-1} culture filtrate, regardless of the time of growth (Table 1). At 120 rpm better results were obtained with the highest amount of inoculum (100 million conidia $flask^{-1}$), but toxin production was still lower compared to the production obtained at 80 rpm (Table 1). The best fungal growth, in terms of fresh and dry weights (3.56 and 0.41 g $flask^{-1}$, respectively) was obtained at 120 rpm after five days with highest amount of inoculum (Table 1). The fungus performed well even when grown for 10 days under these latter conditions. At the lowest inoculum used both the toxin content and the fungal growth were lowest, regardless of the growth time and speed (Table 1). The statistical analysis (Table 2) shows that, considering each of the three single parameters (speed, time and inoculum) only inoculum concentration had a highly significant effect on both growth and toxin production. Growth time was significant (Table 2) only for pH and ascaulitoxin content, whereas speed was significant on the growth characteristics. When considering coupled parameters (Table 2) the combination of speed × inoculum proved to be highly significant on all the growth and metabolic aspects. The combination of the three factors (Table 2) had a significant influence on the toxin production.

3.2.2. Effect of additives and inoculation methods

The addition of Tween 80 in the medium did not increase the toxin content, although it favored mycelial growth (Table 3). Also the addition of glycerol did not increase toxin production or fungal growth.

With regard to the use of different inoculation methods, both chopped mycelium and pre-germinated conidia worked well, but not significantly better than the "standard" use of conidia (Table 3).

3.2.3. Effect of nutritional elements

The two sets of experiments showed that nutritional elements can strongly affect both the growth of the fungus and the production of metabolites. The use of rich nitrogen sources, such as yeast extract, peptone and tryptone all had very positive effects on the biosynthesis of the toxins (Tables 4 and 5). The amount of ammonium tartrate provided in the medium markedly affected the metabolite production, as in the medium with 3× ammonium tartrate toxin production increased, whereas in that with low content the toxin production was almost nil (Table 4). The use of different carbon sources (sorbitol, xylose, glucose, commercial sucrose, or commercial brown sucrose) had slight or nil effects on both fungal growth and metabolism (Tables 4 and 5). The addition of vitamins or of leaf decoction to the media did not influence the growth or toxin production (Tables 4 and 5). Finally, when the initial pH of the medium was increased to 8 or was left unmodified (around 4) both the growth and metabolite production were severely reduced compared to the standard medium, which provided an initial pH adjustment to 5.8 (Table 4).

3.2.4. Use of hydrogel

When grown in hydrogel the fungus was able to produce and release in the culture medium a total of 65 mg of toxin mixture per liter, composed of 30.2 ± 2.5, 12.7 ± 1.8 and 22.1 ± 2.3 mg/l of ascaulitoxin, aglycone and trans amino proline, respectively.

3.3. Fermentation scale-up

At the end of the first fermentation run, lasting ten days, the toxin mixture content in the culture filtrate amounted to a total of 24 $mg\,l^{-1}$ (data not shown). In the second fermentation the final content of toxin mixture increased slightly, reaching 28 $mg\,l^{-1}$ (data not shown). The third production went considerably better, reaching a total around 170 $mg\,l^{-1}$ after 15 day (Fig. 1). The toxin content reached around 60 $mg\,l^{-1}$ (around 25, 15 and 20 $mg\,l^{-1}$ of ascaulitoxin, aglycone and aminoproline, respectively) already after 5 days growth (Fig. 1). The mixture continued to be accumulated almost linearly up to 12 days, with a further increase at 15, without apparently reaching a plateau. During the fermentation, pH reduced progressively from 5.4 to less than 4.6 (Fig. 1).

Table 1
Effect of inoculum quantity, shaking speed and growth time on fungal growth and toxin production.

Speed (rpm)	Time (days)	Inoculum (M conidia)	pH	F.W. (g)	D.W. (g)	Ascaulitoxin (mgl^{-1})	Aglycone (mgl^{-1})	Aminoproline (mgl^{-1})	Total (mgl^{-1})
80	5	1	4.60	0.32	0.09	3.90	nd	3.33	7.23
80	5	10	4.19	0.79	0.15	95.90	52.77	80.77	229.43
80	5	100	4.18	1.09	0.20	48.43	17.27	26.60	92.30
80	10	1	4.45	0.27	0.09	20.43	10.27	14.00	44.70
80	10	10	3.98	0.93	0.15	99.47	48.90	75.90	224.27
80	10	100	3.95	1.73	0.20	49.67	19.03	31.13	99.83
120	5	1	4.97	0.52	0.09	32.07	16.20	20.13	68.40
120	5	10	4.59	0.95	0.16	40.63	12.77	28.77	82.17
120	5	100	4.09	3.56	0.41	88.43	39.10	65.37	192.90
120	10	1	4.83	0.95	0.10	69.50	29.27	41.30	140.07
120	10	10	4.38	1.20	0.19	81.93	29.80	51.97	163.70
120	10	100	4.01	2.80	0.43	86.97	29.00	54.23	170.20

Table 2
Significance of the different cultural conditions (shown in Table 1) on fungal growth and toxin production.

	D.W.	F.W.	pH	Ascaulitoxin	Aglycone	Aminoproline	Total toxins
Speed	***	***	***	***	n.s.	n.s.	*
Time	n.s.	n.s	***	***	n.s.	*	**
Inoculum	***	***	***	***	***	***	***
Speed × time	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
Speed × inoculum	***	***	***	***	***	***	***
Time × inoculum	n.s.	n.s.	n.s.	**	n.s.	*	*
Speed × time × inoculum	n.s.	***	n.s.	n.s.	*	*	*

Level of significance: ***<0.001 < **<0.01 < *<0.05 < not significant.

Table 3
Effect of additives and inoculation method on fungal growth and toxin production.

Inoculum*/additive**	pH	F.W. (g)	D. W. (g)	Ascaulitoxin (mgl^{-1})	Aglycone (mgl^{-1})	Aminoproline (mgl^{-1})	Total toxins (mgl^{-1})
Conidia/none	4.23 bc	1.81a	0.25 a	65.3 b	27.6 a	35.4 b	128.3 b
Conidia/Tween	4.00 ab	5.60 b	0.42 b	31.0 a	16.4 a	21.3 a	68.7 a
Conidia/glycerol	4.33 c	2.31 a	0.28 a	64.9 b	28.1 a	36.8 ab	113.3 b
Chopped mycelium/none	4.11 bc	3.00 a	0.49 b	38.5 a	19.5 a	25.4 a	66.6 a
Pregerminated conidia/none	3.84 a	5.99 b	0.70 c	74.6 b	30.2 a	44.9 b	134.7 b

* Compared to the standard M1-D medium.
** See Section 2 for the experimental procedures different letters along columns mean values statistically different at $p < 0.05$.

Table 4
Effect of medium composition on fungal growth and toxin production (1st experiment).

Medium[a]	pH	Total toxins (mgl^{-1})
Standard medium	4.44	86.90
Standard medium with pH modified to 8.00	4.72	31.90
Standard medium with unmodified initial pH	4.68	28.00
Commercial brown sugar [b]	4.56	43.00
Glucose [b]	4.69	52.00
Commercial sucrose for cooking [b]	4.72	17.90
Fructose [b]	4.67	175.50
Ammonium tartrate (1/5 [c])	3.97	6.90
Ammonium tartrate (3× [c])	4.93	116.00
Tryptone [d]	5.20	283.70
Peptone [d]	5.90	163.30
Yeast extract [d]	5.23	447.70
Vitamins (tiamin and biotin) [e]	4.66	40.70

[a] All the media were prepared by using the standard M1-D with the addition of Tween 80 (0.1%) with the addition/replacement of ingredients as specified.
[b] Replacing saccharose present in the standard medium, at the same quantity.
[c] Amount referred in comparison to the standard recipe.
[d] Replacing ammonium tartrate at the same quantity.
[e] In addition to the standard recipe, at 5 mg/l.

3.4. Toxins analysis and quantification

The culture filtrates obtained in all the experiments described in Section 2 were analyzed by using the method there described for the quantification of each of the three toxins present. Data of toxin contents are reported in Tables 1 and 3–5, and in Fig. 1. The new method for toxin analysis and quantification recently developed using a simple approach of HPLC associated with gel-filtration chromatography (Avolio et al., 2011) proved to be fast and accurate, and suitable to analyze a large number of samples. The detected ascaulitoxin content ranged between 3.9 and 102 mgl^{-1} of culture filtrate (Tables 1 and 5, respectively). The aglycone content ranged between 2.3 and 52.7 mgl^{-1} (Tables 5 and 1, respectively). Finally, aminoproline was detected in a range between 3.3 and 80.7 mgl^{-1} (Table 1). The toxins were well separated by HPLC-gel filtration giving sharp peaks with significantly different retention times using ultrapure water H_2O as eluent and UV-detection.

4. Discussion

Weed control is an activity of particular importance in the management of areas subject to residential and industrial use, such as green areas or public streets and the borders along railway tracks. In those areas the use of chemical herbicides is often severely restricted or even forbidden, due to the potential risks for human activities. The development of procedures for the production and supply of natural substances having herbicidal activity with a lower impact on the environment and reduced or no risks for animals and humans is well regarded and mostly welcomed, although difficult to achieve. The research project and pre-industrial development

Table 5
Effect of medium composition on fungal growth and toxin production (2nd experiment).

Medium[1]	Time (days)	pH	D.W. (g)	F.W. (g)	Ascaulitoxin ($mg l^{-1}$)	Aglycone ($mg l^{-1}$)	Aminoproline ($mg l^{-1}$)	Total ($mg l^{-1}$)
Standard medium + Tween 80	6	4.68 b	0.82 a	2.25 a	36.1 e	15.2 c	29.6 e	80.9 e
Tryptone[3]	6	6.45 c	1.57 c	10.48 e	10.1 a	2.3 a	6.5 a	18.9 a
Yeast extract[3]	6	7.99 d	1.75 c	11.46 e	102.7 h	50.1 f	65.3 g	218.1 h
Sorbitol[2]	6	4.94 b	0.76 a	2.04 a	10.0 a	nd	6.4 a	16.4 a
Xylose[2]	6	4.72 b	0.73 a	1.92 a	28.4 c	11.6 b	20.5 bc	60.5 bc
Standard medium + Tween 80	10	3.85 a	0.70 a	5.75 c	39.5 f	20.4 d	32.6 f	92.5 f
Sugar for cooking[2]	10	3.99 a	0.48 a	3.59 b	28.5 c	12.4 b	23.1 cd	64.0 c
Commercial ammonium tartrate[3]	10	3.92 a	0.58 a	2.82 ab	42.1 g	24.6 e	35.2 f	101.9 g
Fat hen leaf infusion (2 g/l)[4]	10	3.67 a	0.90 a	8.39 d	25.2 b	11.0 b	17.8 b	54.0 b
Fat hen leaf infusion (10 g/l)[4]	10	3.50 a	1.26 b	13.73 f	31.3 d	16.4 c	25.7 d	73.4 d

[1] All the media were prepared by using the standard M1-D with the addition of Tween 80 (0.1%) with the addition/replacement of ingredients as specified.
[2] Replacing saccharose present in the standard medium, at the same quantity.
[3] Replacing ammonium tartrate at the same quantity.
[4] In addition to the standard recipe.

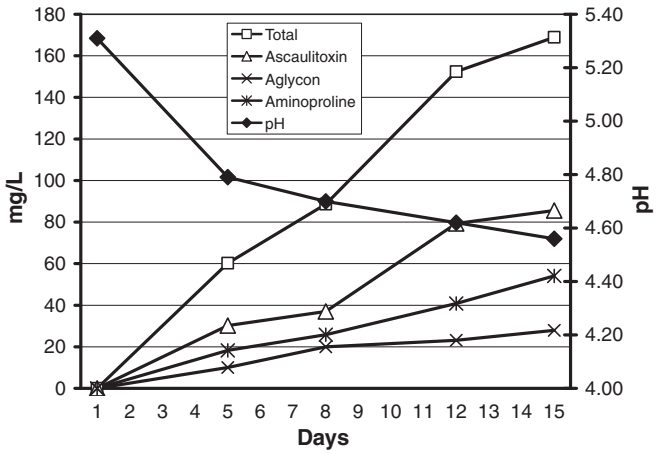

Fig. 1. Time course of toxin production and pH variation in the 3rd fermentation run.

presented here aims to develop production of a new natural herbicide (i.e. the mixture of toxins) already identified in its active components, to control weeds. This product could complement or replace synthetic products currently in use, reducing environmental impact.

Besides effectiveness, one of the major factors influencing the competitiveness of a natural herbicide in respect to synthetic ones is the cost of production, i.e. fermentation and purification. And the cost of nutrients used to grow the organisms is undoubtedly one of the most important. Different tests have been performed in order both to increase the capacity of AC1 to produce the toxic mixture (e.g. with the use of additives), or to lower the costs of the substrates prepared (i.e., by using commercial substances rather than pure laboratory chemicals).

The quantification of toxin production was made by using a new analytical method, based on the use of HPLC associated with gel-filtration chromatography. The method appears to be simple, rapid and inexpensive, compared to the two methods (Evidente et al., 2001; Fiore et al., 2010) previously developed for the analysis of *A. caulina* toxins. The method appears suitable even for the analysis of toxins at trace levels, being very sensitive with detection limits in the range 0.001–0.0005 μg. Furthermore, the toxins are eluted in a few minutes (between 5 and 9 min), and the method is suitable for routine analysis applicable to complex samples such as the mixture of toxins obtained from fungal culture filtrates. Finally, the method is extremely precise, as overlaps of the toxin peaks with other metabolites have never been observed and the recovery of all three toxins was near to 100%.

An analysis of the composition and costs of the liquid medium routinely used in the laboratory for the growth of AC1 showed that the three main components affecting the overall costs of the substrate are sucrose, ammonium tartrate and magnesium sulfate, by 67%, 24% and 6%, respectively. The successful use of similar nutrients having a different purity grade or obtained from different sources (e.g., alimentary sucrose or fertilizers) would allow dramatic reduction of production costs, by a factor estimated to approach 1000 (data not shown).

The fungus proved to be suitable for growth in shaken conditions, and performed promisingly well in the pilot fermentation system used. This suggests the possibility of scaling up the fermentation to larger fermentation systems, making it suitable for industrial fermentation. Of course, a number of further aspects should be considered before this can be realized which will require more specifically oriented funds.

The initial lab growth conditions were far from suitable for an industrial application, involving growth for 4 weeks in static conditions. The preliminary observations had shown the potential of AC1 to be developed at a semi industrial scale, showing its capability for growth in shaken conditions, and for shorter periods, in contrast to other microbes routinely used in fermentation processes, such as yeast, bacteria, or even yeast-like fungi. In static conditions the fungus slowly produces a dispersed mycelial mass, that after some days, becomes dense and compact, floating on and covering of the entire medium surface. This was not suitable for shaken conditions in flasks, and even less suitable in the fermenter, as the presence of a dense mass could block the rotation of the blades. Thus, it was necessary to produce small mycelial fragments dispersed in the whole medium. This was obtained and successively improved on by increasing the amount of germination conidia used as inoculum and by adding a non ionic surfactant (Tween 80) to the medium, in order to keep the fragments separated, and prevent their adhesion to the flask glass. To our knowledge, this is the first reported scaling up of *Ascochyta* fungal cultivation to a pilot production system by using a stirred tank fermenter. This process development involved the optimization of both nutritional and engineering parameters. Composition of nutritional medium, pH and temperature but also engineering aspects like aeration, agitation and medium sterilization become critical as a microbial process is scaled up to the production level (Masurekar, 2008). A further improvement was obtained in the fermenter by increasing the speed to high rpm (higher than 200) for a short time (1–2 min) every 2 or 3 days. This allowed the mycelial fragments adhering on the fermenter blades and walls to return into suspension and continue to grow, and also to keep the system functioning. An important aspect to be further considered is the speed of rotation. While rotation is fundamental in a fermentation system, the fungus proved to be very sensitive to the high rpm, which reduced its growth.

In general, the analysis of all the data presented in Table 5, grouped according to the speed, showed that higher speed

provided a better fungal growth and toxin accumulation. In this latter case the total toxin content raised from an average of 116 to 136 mg l^{-1} considering all the data grouped (data not shown). However, the best performance was obtained when growing the fungus for a longer time (10 days) at slower shaking speed. This could be due to a better capability of the fungus to properly utilize the nutrients and the oxygen available in the medium. Among the factors that determine the morphology and the general course of fungal fermentations, the amount, type (spore or vegetative) and age of the inoculum are of primary importance. In earlier attempts made to standardize inoculum for citric acid production in submerged culture, van Suijdam et al. (1980) reported that *Aspergillus niger* pellets would only form at inoculum sizes below 10^8 spores ml^{-1}, while according to Calam (1987), *Penicillium chrysogenum* forms pellets at inoculum sizes below 10^4 spores ml^{-1}. In the latter case, penicillin production in production flasks increased ten times as inoculum size increased from 10^2 to 10^4 spores ml^{-1}. In our case, the best toxin contents were obtained when using a medium/high amount of conidial inoculum (10^5–10^6 conidia ml^{-1}) (Table 1), and much lower amounts of toxin with a low inoculum (10^4 conidia ml^{-1}). A possible explanation is that an excess of conidia in a flask could increase the precipitation of the conidia at an early stage of their germination, resulting in a reduced number of propagules, or even in a higher competition of the mycelial fragments in terms of space and availability of nutrients (Papagianni, 2004).

With regard to the use of different nutrients, not all of them proved to be suitable for fungal growth. For example, in general the use of carbon sources different from sucrose did not favor fungal growth, and in some cases the mycelium turned brown after only a few days, stopping its growth. Conversely, some other nutrients proved to speed up the growth or to sustain it for a longer period of time. Among them, yeast extract performed very well, allowing a higher toxin yield (Tables 4 and 5).

Considering the best performances obtained in the fermentations when using higher amount of inoculum, the availability of a fast system that allows production and easy collection of large quantities of conidia appears an important element of the whole optimization process. The use of the cellophane disks placed on PDA plates allowed led to a better yield compared to the standard plates. The use of wheat-bran base provided a much higher yield of conidia that can be easily harvested. They could be routinely produced, and even stored, retaining a high germination rate over time (data not shown).

With regard to the use of hydrogels, the fungus proved to grow well. After harvesting, pellets were collected and placed on PDA plates, where they very promptly developed hyphae that quickly started producing pycnidia. The production of the toxin mixture was comparable to the production obtained in the more traditional systems (static and liquid free mycelium). The system appears promising and susceptible of a number of improvements. First of all, the number of propagules could be increased, allowing a more favorable ratio between amount of growing mycelium and nutrients. Secondly, the size of the hydrogels could be optimized, finding the size most suitable for nutrient use. Third, the growth could probably be prolonged for a longer time, allowing further synthesis and accumulation of the toxins. The system of growth encapsulated in hydrogels seems to be compatible with a slow-rotating fermentation system, favoring the use of the nutrients and preventing the formation of long free hyphae, that adhere to the walls and to the blades of the fermenter, hindering the fermentation process.

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