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ISOLATION AND CHARACTERIZATION OF TENUAZONIC ACID PRODUCED BY *ALTERNARIA ALTERNATA*, A POTENTIAL BIOHERBICIDAL AGENT FOR CONTROL OF *LANTANA CAMARA*

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Abstract: Microbial preparation of herbicide is defined as bioherbicide that can control the weed. In this approach, indigenous plant pathogens isolated from weeds are cultured to produce the large numbers of infective propagules which are applied at a rate that will cause high levels of infection leading to suppression of the target weed. During the present investigation, cell free culture filtrate (CFCF) of *Alternaria alternata* was evaluated for its phytotoxicity against a noxious weed *Lantana camara*. The results of cut shoot, seedling and detached leaf bioassays revealed the presence of a toxic metabolite in the CFCF and a significant reduction in chlorophyll and protein content were also noticed. Phytotoxic moiety was further purified and characterized by using solvent partition, thin layer chromatography (TLC), FTIR and ^{1}H NMR analysis. The acetone extract induced maximum phytotoxic damage at a concentration of 100 μg/ml and TLC purified fraction also exhibited herbicidal potential. The toxic compound was identified as tenuazonic acid upon comparison with FTIR and ^{1}H NMR spectra. This is the first evidence that confirmed the herbicidal potential of a biorational, tenuazonic acid was produced by submerged fermentation of *A. alternata*.

Key words: *Alternaria alternata*, *Lantana camara*, mycoherbicide, tenuazonic acid, phytotoxin, bioherbicide

INTRODUCTION

Weeds cause serious ecological and economical problems and are capable of altering the processes in ecosystem, displacing the native flora and fauna. *Lantana camara* of family Verbenaceae is listed amongst the worst weed in the world. *L. camara* has become a serious problem for tropical countries, where it invaded millions of hectares of land. Prolific seed production and easy dispersal helped to becomes a pest of serious dimensions. It is established and expanding in many regions of the world, often as a result of clearing of forest for timber or agriculture. *L. camara* is posing a serious problem in forestry plantations, chokes all other vegetation and becomes the dominant species (Sahu and Singh 2008). With ever increasing population of *L. camara* in both urban and rural localities, the associated problems are also growing enormously. The plant is toxic to humans and the ruminant livestock and therefore demands immediate management through ecofriendly methods.

Biological control is a deliberate use of natural enemies to suppress the growth or reduce the population of weed species. During last two decades biological control received a considerable attention. Those have been results of the intensive use of chemical herbicides coming under scrutiny due to an increasing number of resistant or tolerant weeds, effect of non-target organisms, contamination of soil, ground water and food etc. Microbial secondary metabolites appear to be a lucrative source of novel structures having unique mode of action which could be exploited as commercial herbicides (Kenfield *et al.* 1988; Abbas and Duke 1995). Exploitation of phytotoxic properties of microbial metabolites attracted attention of a large number of researchers involved in weed management programme (Fischer and Bellus 1983; Duke 1986).

In the present investigation, we studied the mycoherbicidal potential of secondary metabolites obtained from the fungal biocontrol agent *Alternaria alternata* as ecofriendly herbicides for management of the noxious weed *L. camara*. From secondary metabolites of the fungus *A. alternata* various phytotoxic effects were reported during pathogenesis, including chlorosis or necrosis on leaves and other parts of the plant as well as inhibition of seed germination (Liakopoulou-Kyriakides *et al.* 1997).

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MATERIALS AND METHODS

Fungal strain

A strain of the test fungus *A. alternata* was isolated from diseased leaves of the target weed, *L. camara*. It was grown on potato dextrose agar medium (PDA: extract of 200 g peeled potatoes, 20 g dextrose, 20 g agar and distilled water up to 1 000 ml) at 25±1°C and maintained at 4±1°C in a refrigerator for further studies.

Production and extraction of Cell Free Culture Filtrate (CFCF)

5 mm disc of the test strain, grown on PDA medium for 7 days at 25±1°C in the glass petri plates were inoculated into 250 ml Erlenmeyer's flasks containing 100 ml of Richard's broth (10 g KNO_3, 2.5 g $MgSO_4$, 2.5 g KH_2PO_4, 30 g sucrose and distilled water up to 1 000 ml) and were incubated at 25±1°C in B.O.D incubator (Yorco, India) as stationary culture.

Under aseptic conditions the metabolized growth medium was filtered through a pre-weighed Whatman filter paper No. 1 after 7, 14, 21 and 28 days of incubation and then centrifuged at 4 000 rpm for 10 min. The pellet was discarded and the supernatant was again passed through the Sartorius 0.45 µm, Minisart (Sartorius, Gottingen, Germany) under *in vacuo* conditions to obtain the final CFCF (Vikrant *et al.* 2006).

Screening of phytotoxicity of CFCF

Shoot cut bioassay

Shoots of the weed were taken from healthy 8 weeks old plant and dipped in the CFCF of 7, 14, 21, 28 days old fermented broth at concentration of 25, 50, 75 and 100% in glass vials and these vials were capped with foil to make them air tight and placed in plant growth chamber (Yorco, India) at 26±1°C. The effects were observed after 24, 48 and 72 hours of treatment (Sharma *et al.* 1969; Chiang *et al.* 1989). Phytotoxicity was determined by following the method of Abbas and Boyette (1992) using a rating scale of 0–5 (0-no symptoms, 1-slight chlorosis and lower leaf drop, 2-marked chlorosis and slight necrosis, 3-acute chlorosis and marked necrosis, 4-strong chlorosis and strong necrosis, 5-acute chlorosis and necrosis leading to death of shoots).

Seedling bioassay

Seedlings were raised in pots containing soil, sand and peat (1 : 1 : 1). Different dilutions (25, 50, 75 and 100%) of the toxic metabolites obtained from 7, 14, 21 and 28 days fermented broth were made and seedlings were sprayed to run off condition and were incubated for different periods. Observations regarding toxicity for the seedlings were made regularly on a 0–5 rating scale as described above (Abbas *et al.* 1992).

Detached leaf bioassay

Leaves detached from the plant were surface sterilized with 2% NaOCl. The area of approximately 3 mm^2 on the upper leaf surface was gently scratched and 20 µl of the toxin was injected by using sterilized syringe. The leaves were than incubated on a sterilized moist chamber (made in 9 cm petri plates using cotton and filter paper) under continuous fluorescent light at 26±1°C (Sharma *et al.* 2004). The effect was observed after 6, 12 and 24 h at room temperature.

In all bioassays described above, sterilized unmetabolized growth medium was taken as control and the sterilized distilled water was taken as control over control (Sharma *et al.* 2004) and the experiments were carried out in triplicates.

Reduction in chlorophyll and protein content of *L. camara* after all bioassays were screened to determine the mode of action of phytotoxin. The chlorophyll content was determined on a pre-weight basis by the method of Arnon (1949) and total protein content was determined by the method suggested by Lowry *et al.* (1951).

Thermal stability of the phytotoxic moiety

To assess the mode of extraction of the phytotoxic moiety, it was extremely essential to ascertain the thermal nature of phytotoxin. The CFCF was subjected to different temperature treatment: 50, 100 and 121°C (autoclaved). Each treatment was carried out for 15 minutes. The phytotoxic activity of each treatment was assessed using shoot cut bioassay (Siddaramaiah *et al.* 1979). Each treatment was carried out in triplicates and untreated CFCF at room temperature taken as control.

Purification and characterization of the phytotoxic moiety

Extraction

The CFCF was obtained as described earlier and concentrated four times of the original volume (Krishnamohan and Vidhyasekaran 1989; Brain *et al.* 1945). This was further subjected to organic solvent extraction. Solvents used for the extraction procedure were hexane, petroleum ether, carbon tetrachloride, chloroform, acetone and ethyl acetate.

Assessment of biological activity

All layers obtained in solvent extraction were subjected to *in vacuo* dessication at 40°C to remove solvent and to obtain residues. Residues were named as obtained: Fraction 1 (hexane) and Fraction 2 (petroleum ether), respectively. The test residues were prepared as stocks using 5 ml distilled water and were tested for their phytotoxic activity using detached leaf bioassay (Strobel 1973; Karr *et al.* 1974; Sugawara *et al.* 1985).

Thin layer chromatography (TLC)

Thin layer chromatography of phytotoxic fraction was performed on silica gel (0.25 mm) with toluene: ethyl acetate: formic acid (6 : 3 : 1). The plates were allowed to run for about 75 minutes. Spots were visualized by spraying with 1% ninhydrin in acetone and R_f of each band was calculated. The plates were dried at 40°C for 6 hours in an oven and scooped out the band, and then it was eluted with acetone and further tested for its phytotoxic activity against *L. camara* by detached leaf bioassay. The samples were analyzed in triplicates.

FTIR and ^{1}H NMR analysis

The dry TLC fraction eluted with acetone was filtered through the Sartorius filter paper. The filtered sample was evaporated on a hotplate (Remi, India) at 40°C till dryness. The dry powder was mixed with purified salt KBr. This powder mixture was then crushed properly in order to ensure that the pellet becomes translucent.

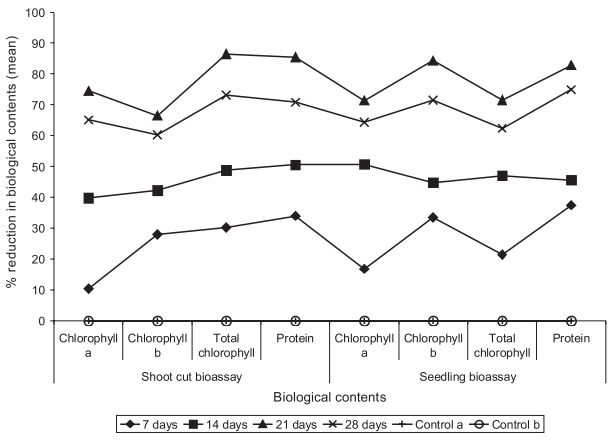
The FTIR analysis was carried out by using Shimadzu FTIR-8400, SCE model, and scanned against KBr blanks using DRS mode (DRS-8000), resolution-4 with the number of scans 20.

High-resolution ^{1}H nuclear magnetic resonance (NMR) spectra were obtained by using a Bruker AV-400 (Bruker 400 MHZ) spectrometer (Bruker Biospin, Switzerland).

RESULTS AND DISCUSSION

Phytotoxin production

It is evident from data presented in figure 1 that CFCF obtained from fermented broth of *A. alternata* had a varied degree of toxicity against *L. camara*. Maximum toxicity was recorded when shoots and seedlings were treated with CFCF obtained from 21 days old fermented broth. The initial symptoms observed within 6 h of treatment on the affected shoots and seedlings were prominent wilting and slight curling. At advanced stage rapid browning of stem, wilting of leaves, and veinal necrosis of leaves was observed. Leaves exhibited acute necrosis leading to death of shoots and seedlings.



Control a: uninoculated Richard broth
Control b: distilled water
Incubation temperature: 26±1°C
Medium: Richard broth

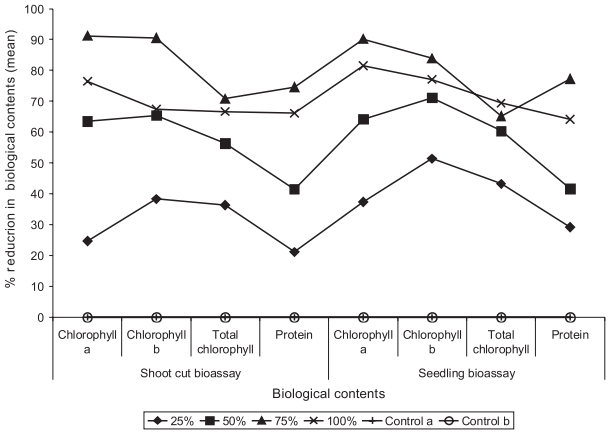
Fig. 1. Reduction in biological content in a shoot cut and seedling bioassay of *L. camara* by treating with CFCF of *A. alternata* of different incubation days

Significant reduction in photosynthetic pigment and protein contents was also recorded. Maximum reduction i.e., 74.5, 66.5, 86.4 and 85.4% was reported for chlorophyll a, chlorophyll b, total chlorophyll and protein, respectively when shoots were treated with CFCF obtained from 21 days old fermented broth followed by CFCF obtained from 28, 14 and 7 days old fermented broth.

Similarly, when seedlings were sprayed with CFCF obtained from 21 days old fermented broth, maximum reduction was observed in biological contents viz., 82.8, 84.3, 71.5 and 71.4% for chlorophyll a, chlorophyll b, total chlorophyll and protein, respectively. Thus it is evident from the above discussion that the fungal pathogen produced maximum toxin at 21 days of incubation.

Phytotoxicity at different concentrations

Data presented in figure 2 shows that the phytotoxic damage was directly proportional to the concentration of CFCF. Effect of different dilutions of CFCF obtained from 21 days old fermented broth was determined for their phytotoxicity against *L. camara* by shoot cut and seedling bioassay. Maximum damage was recorded when shoots and seedlings of the test weed were treated with 75% concentration followed by 100, 50 and 25% concentration of CFCF. At 75% concentration initial symptoms of phytotoxicity were characterized by the appearance of necrotic patches within 6 h of application with advancement in treatment time, severe necrosis was observed and at advanced stage, curling and wilting was recorded.



Control a: uninoculated Richard broth
Control b: distilled water
Incubation temperature: 26±1°C
Medium: Richard broth

Fig. 2. Reduction in biological content in a shoot cut and seedling bioassay of *L. camara* by treating with CFCF of *A. alternata* of different concentrations

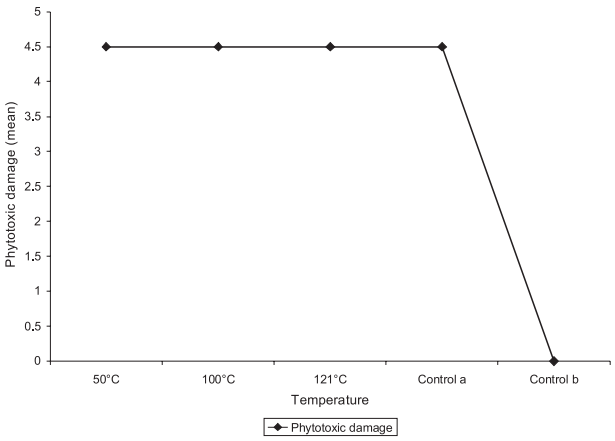
Drastic reduction in photosynthetic pigment and protein was also observed in shoots and seedlings treated with CFCF at 75% concentration. A maximum reduction of 91.2, 90.5, 70.8 and 74.6% for chlorophyll a, chlorophyll b, total chlorophyll and protein, respectively for shoots and 90.2, 83.9, 65.2 and 77.2% for chlorophyll a, chlorophyll b, total chlorophyll and protein, respectively for seedlings were reported at 75% concentration of CFCF obtained from 21 days old fermented broth.

Observations regarding phytotoxicity of fungal secondary metabolite obtained in the present investigation are in accordance with those recorded by many other workers working on biological activity of secondary metabolites from fungal extracts (Walker and Templeton 1973; Hoagland 1990; Pandey *et al.* 1992; Boyette and Ab-

bas 1995; Mishra *et al.* 1996; Saxena *et al.* 2000). Variation in biological activity of the phytotoxin in treated shoots and seedlings have also been recorded by several workers (Pandey *et al.* 2000; Joseph *et al.* 2002; Thapar *et al.* 2002; Pandey *et al.* 2003).

Thermal stability of phytotoxin

As evident from the data presented in figure 3 the phytotoxin was stable at 50, 100, and 121°C. Thus it could be concluded that the phytotoxic moiety was thermo-tolerant and thermo-stable. Ros *et al.* (1993), reported similar observations during production of rhizopin from *Rhizopus nigricans*. Kurian *et al.* (1977) also reported thermal stability and non-proteinaceous nature of a toxin produced by *Cristulariella pyramidalis*.



Control a: CFCF at room temperature
Control b: uninoculated Richard broth at room temperature
R.H..: 80%
Room temperature: 26±1°C

Fig. 3. Thermal nature of phytotoxin from *A. alternata*, phytotoxicity rating against the detached leaves of *L. camara*

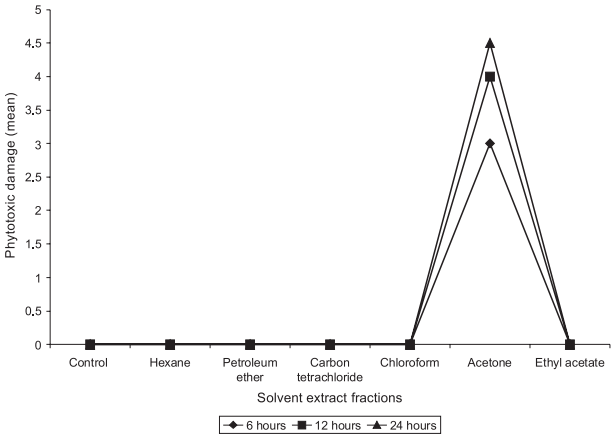
Purification and characterization of phytotoxin

Solvent extraction and detection of phytotoxicity

The fractions so obtained from the solvent extraction were vacuum evaporated (Rotary evaporator, MAC, India) at 40–45°C and their residues were tested using detached leaf bioassay to detect and isolate a phytotoxic moiety. 100 µg/ml concentrations of residues were tested during the detached leaf bioassay. It was found that fraction 5 (acetone extract) induced phytotoxic damage after 6 hours post treatment (hpt) during the detached leaf bioassay; this is depicted in figures 4 and 5. Other fractions did not induce phytotoxic symptoms. Robeson *et al.* (1991) extracted tenuazonic acid from CFCF of *A. alternata* by employing ethyl acetate. Similarly Yoshida *et al.* (2000) isolated toxic compounds from the CFCF of *Colletotrichum dematium* by fractionating it with an equivalent volume of n-hexane and ethyl acetate.

Thin layer chromatography

Phytotoxin fraction 5 (acetone extract) was further clarified for characterizing the phytotoxic moiety. The analytical technique TLC was employed. A blue spot was visualized by spraying with 1% ninhydrin in acetone (Fig. 6). The R_f obtained was 0.5–0.6. Herbicidal potential of TLC purified fraction against the detached leaves of *L. camara* is shown in Figures 5 and 7. Similar results of TLC based toxin purification were observed and reported by Davis *et al.* (1977).



Control: distilled water
R.H..: 80%
Room temperature: 26±1°C

Fig. 4. Herbicidal potential of phytotoxic solvent extract fractions of CFCF of *Alternaria alternata* against the detached leaves of *Lantana camara*

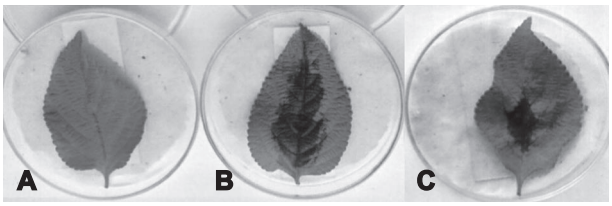


Fig. 5. Detection of phytotoxicity by detached leaf bioassay
A: Control: uninoculated Richard broth (no symptoms)
B: acetone extract (high necrosis)
C: TLC purified fraction (marked necrosis)

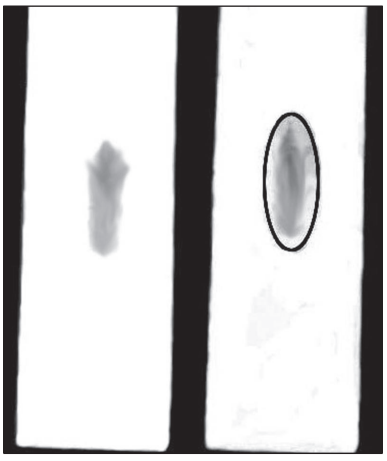
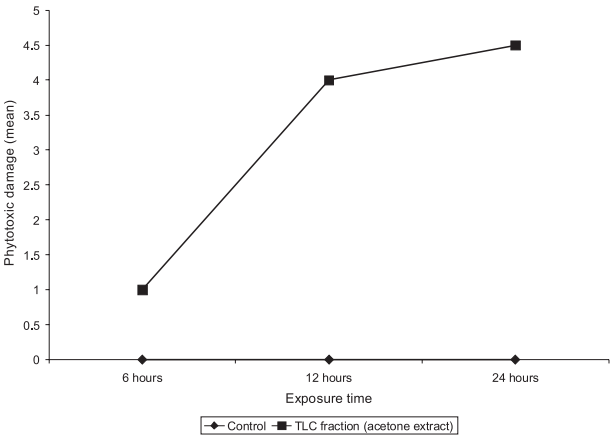


Fig. 6. TLC plate of the phytotoxic fraction, circle shows the blue spot (R_f: 0.5–0.6)



Control: distilled water
R.H..: 80%
room temperature: 26±1°C

Phytotoxic damage scoring for figure 3, 4 and 7:
0: 0 – 4% plant death (no effect)
1: 2 – 19% plant death (slight chlorosis & lower leaf drop)
2: 20 – 49% plant death (marked chlorosis and slight necrosis, drooping of entire twings)
3: 50 – 79% plant death (acute chlorosis and marked necrosis)
4: 80 – 94% plant death (high chlorosis and high necrosis)
5: 95 – 100% plant death (acute chlorosis and necrosis)

Fig. 7. Herbicidal potential of TLC purified fraction of CFCF of *Alternaria alternata* against the detached leaves of *L. camara*

FTIR and ^{1}H NMR analysis of the phytotoxic band separated by TLC

The FTIR spectrum (Fig. 8) of the sample (acetone extract) gave the following signals and assignments:

1. OH stretching: – 3 500–3 560 cm (concentrating samples broadens the band and moves it to 3 000 cm).
2. Aliphatic C-H stretching: – 1 380 cm (weak), 1 260 cm (strong) and 2 870, 2 960 cm (both strong to medium).
3. Presence of – C = O, Keto group, cyclic membered: – 1 720 cm.
4. Presence of primary amine: – doublet between 3 400–3 500 cm and 1 560–1 640 cm (strong).

The ^{1}H NMR spectrum (acetone) gave the following signals and assignments: 0.9 ppm (d + t overlapping) of 2 x CH_3, 1.3 ppm (m) of CH_2, 2.0 ppm (m) of CH_2, 2.5 ppm (s) of $COCH_3$, 3.7 ppm (d) of = CO-CH-N, 6.8 ppm (broads) of NH, and 9.3 ppm (broads) of OH.

The herbicidal compound was identified as tenuazonic acid (Fig. 9) upon comparison with the IR spectra reported by Davis *et al*. 1977 and comparison of the above properties with those reported in the literature (Stickings *et al.* 1959; Kaczka *et al.* 1964; Mikami *et al.* 1971; Meronuck *et al.* 1972; Steyn *et al.* 1976).

In conclusion, it is apparent from the present investigation that the phytotoxin (tenuazonic acid) elaborated from *A. alternata* is novel and it exhibited potential to be exploited as a natural herbicide for the target weed *L. camara.* The present investigation provides us a break through for replacement of synthetic chemicals without incurring huge economic losses.

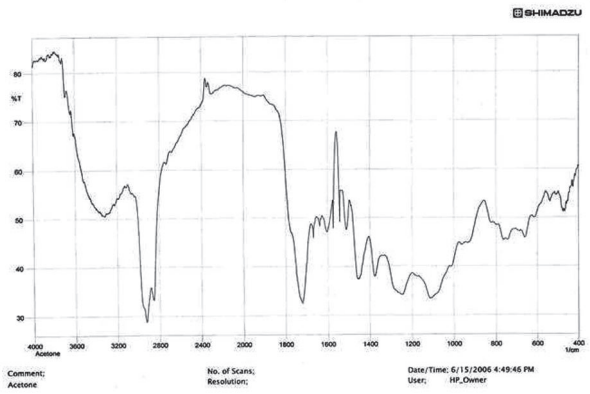


Fig. 8. FTIR spectrum of tenuazonic acid

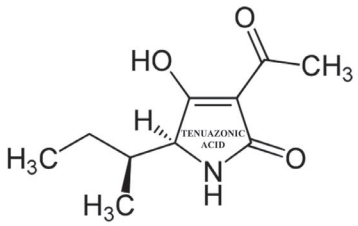


Fig. 9. Structure of tenuazonic acid (Davis *et al*. 1997)

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POLISH SUMMARY

IZOLACJA I CHARAKTERYSTYKA KWASU TENAUZOWEGO WYTWORZONEGO PRZEZ *ALTERNARIA ALTERNATA*, POTENCJALNEGO HERBICYDU DO ZWALCZANIA *LANTANA CAMARA*

Herbicyd mikrobiologiczny jest określany jako bioherbicyd, który może zwalczać chwasty. Rodzime patogeny roślin wyosobnione z chwastów są masowo hodowane w celu uzyskania dużych ilości jednostek propagacyjnych, mogących wywoływać znaczące infekcje prowadzące do supresji docelowego chwastu. W badaniach oceniano fitotoksyczność bezkomórkowych filtratów kultur *Alternaria alternata* (CFCF) wobec szkodliwego chwastu *Lantana camara.* Wyniki badań przeprowadzonych na odciętych łodygach siewek oraz biotestu na odciętych liściach ujawniły obecność toksycznego metabolitu w filtracie, a także istotną redukcję zawartości chlorofilu i białka. Fi-

totoksyczna część filtratu została następnie oczyszczona i scharakteryzowana przy pomocy rozdzielenia go rozpuszczalnikami oraz cienkowarstwowej chromatografii (TLC), FTIR i analizy ^{1}H NMR. Ekstrakt akcentowany powodował maksymalne uszkodzenia fitotoksyczne w stężeniu 100 μg/ml, a oczyszczona frakcja TCL wykazywała również potencjał herbicydowy. Toksyczny związek określono jako kwas tenauzonowy, w wyniku jego porównania ze spektrami FTIR i ^{1}H NMR. Jest to pierwszy dowód potwierdzający potencjał herbicydowy badanego związku. Kwas tenauzonowy uzyskano wykorzystując podpowierzchniową fermentację przy użyciu *A. alternata.*