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# Effects of Antibiotics on Contaminated Callus Cultures of Pyrethrum

By

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With 4 Figures

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Summary

BERGANT M., AMBROŽIČ-DOLINŠEK J., DEMŠAR T., DREO T., RAVNIKAR M., ŽEL J. & CAMLOH M. 2005. Effects of antibiotics on contaminated callus cultures of pyrethrum. – Phyton (Horn, Austria) 45 (2): 197 – 206, with 4 figures. English with German summary.

Pyrethrum [*Tanacetum cinerariifolium* (TREVIR.) SCHULTZ-BIP., *Asteraceae*] is a source of pyrethrins, natural insecticides that are widely used in agriculture and for domestic purposes. In callus cultures of pyrethrum microbial contaminants were observed routinely, therefore it was necessary to use antibiotics to maintain aseptic cultures. The antibiotics, streptomycin, cephalexin and penicillin-G were used for antibiotic pulse treatment. Their effect on callus growth and microbial elimination was investigated.

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Callus cultures of two different lines were incubated for 1 or 2 days in liquid medium containing different combinations and concentrations of antibiotics and then cultured for 6 weeks on solid medium without antibiotics. One day of antibiotic treatment had no effect on callus growth. After two days of treatment, however, growth was significantly inhibited. Growth suppression was genotype-dependent and high concentrations of antibiotics were found to be deleterious on callus growth.

PCR analysis with eubacterial 16S rDNA primers was done for detecting bacteria, as it enables low, invisible concentrations of bacteria in callus cultures to be traced. Six weeks after antibiotic treatment no bacteria were detected in line L4 incubated for 2 days in medium containing 360 mg/l streptomycin, 160 mg/l cephalexin and 8 mg/l penicillin G. Lower concentrations of antibiotics, however, exhibited no bacteriostatic effect.

These results indicate that, although the higher concentrations of antibiotics used in pulse treatment greatly reduce callus growth, they can successfully eliminate bacterial contamination from pyrethrum callus cultures.

## Zusammenfassung

Bergant M., Ambrožič-Dolinšek J., Demšar T., Dreo T., Ravnikar M., Žel J. & Camloh M. 2005. Wirkung von Antibiotika auf kontaminierte Kalluskulturen von Pyrethrum. – Phyton (Horn, Austria) 45 (2): 197 – 206, 4 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Pyrethrum [*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip., *Asteraceae*] ist eine Quelle für die Gewinnung von Pyrethrinen, welche natürliche Insektizide darstellen und in der Landwirtschaft und im häuslichen Bereich weit verbreitet sind. In den Kalluskulturen von Pyrethrum können mikrobielle Verunreinigungen immer wieder beobachtet werden, weshalb es sich als notwendig erweist, Antibiotika zu verwenden, um sterile Kulturen aufrecht zu erhalten. Die Antibiotika Streptomycin, Cephalexin und Penicillin-G wurden für eine Einmalbehandlung verwendet. Ihre Auswirkung auf das Kalluswachstum und die Vernichtung von Mikroorganismen wurde untersucht.

Kalluskulturen von zwei unterschiedlichen Linien wurden für ein bzw. zwei Tage in ein flüssiges Medium, welches verschiedene Kombinationen und Konzentrationen an Antibiotika enthielt, inkubiert. Anschließend wurden die Kalli über sechs Wochen auf einem festen Medium ohne Antibiotika kultiviert. Die Behandlung über einen Tag mit Antibiotika hatte keinen Einfluss auf das Kalluswachstum. Eine zweitägige Behandlung allerdings verminderte das Wachstum signifikant. Die Wachstumsverminderung war abhängig vom Genotyp und hohe Konzentrationen von Antibiotika waren schädlich für das Kalluswachstum.

Die PCR-Analyse mit eubakteriellen 16SrDNA Primern wurde für die Erkennung von Bakterien heran gezogen, da sie erlaubt, geringe, nicht sichtbare Konzentrationen von Bakterien in Kalluskulturen ausfindig zu machen. Sechs Wochen nach der Behandlung mit den Antibiotika konnten keine Bakterien in der Linie 4, welche über zwei Tage in einem Medium, das 360 mg/l Streptomycin, 160 mg/l Cephalexin und 8 mg/l Penicillin-G enthielt, nachgewiesen werden. Geringere Antibiotikakonzentrationen hatten hingegen keine bakteriostatische Wirkung.

Die Ergebnisse zeigen, dass, obwohl die höheren Konzentratonen von Antibiotika, welche für die Einmalbehandlung verwendet wurden, das Kalluswachstum

deutlich verminderten, die Antibiotika erfolgreich Bakterienkontaminationen bei Kalluskulturen von Pyrethrum eliminieren können.

## Introduction

Pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.) is an important source of natural insecticides widely used in agriculture and for domestic purposes, and is therefore often cultured in vitro (HITMI & al. 1999, JOVETIĆ & DE GOOIJER 1995). The critical stage in establishing a tissue culture is to obtain cultures free of bacterial contamination. In general, plant material obtained from the field can be heavily infested with microorganisms (TEIXEIRA DA SILVA & al. 2003). In annual plants for example bean, two seed-borne bacteria were identified during seed health testing (GRUM & al. 1998). Perennial species in particular often have endophytic microflora which can become problematic under changed growth condition, such as in the initiation of in vitro culture (PEÑALVER & al. 1994). Bacterial contamination is commonly controlled by antibiotic treatment which can, apart from their bactericidal action, affect the growth and alter the morphogenesis of in vitro cultures (TENG & NICHOLSON 1997, EADY & LISTER 1998, KESKITALO & al. 1998, TENG & TENG 2000). The effect of antibiotics on contaminated cultures varies with the plant species and the antibiotics used. Furthermore, different genotypes of the same species can react differently (KESKITALO & al. 1998).

The antibiotics are usually incorporated in the culture medium at low concentrations for long-term incubation periods (KESKITALO & al. 1998, TAHMATSIDOU & CASSELLS 1997). On the other hand, the rarely studied treatment with a high concentration of antibiotics for a short time (antibiotic pulse treatment-APT) can also be used effectively to avoid contamination of in vitro cultures (TENG & NICHOLSON 1997, TENG & TENG 2000). In general, the combinations of antibiotics were more effective than the use of a single one (KNEIFEL & LEONHARDT 1992).

The objective of our study was to determine whether antibiotic pulse treatment (APT) could eliminate bacterial contamination of pyrethrum callus cultures and whether it would have post-treatment effects on callus growth. To detect bacterial contaminants, PCR analysis with eubacterial 16S rDNA primers was used. To examine genotypic variation in antibiotic sensitivity, two lines of the pyrethrum callus culture were included in the study.

## Material and Methods

### Plant Material

Pyrethrum (*Tanacetum cinerariifolium*) grown in the Botanical Garden of Ljubljana was used as plant material. Callus cultures were initiated from the flower heads of different plants and maintained in ½ MS-medium (MURASHIGE & SKOOG

1962) supplemented with 30g/l sucrose, 8 g/l agar, 20 μM NAA, 2 μM BA and 0.1 g/l ascorbic acid under a 16 h photoperiod (23 ± 2 °C) at 100–110 μmol $m^{-2}$ $s^{-1}$ (fluorescent light tubes), and subcultured every 3 weeks. In our experiments, two callus lines originated from different plants were used.

### Antibiotic Pulse Treatment (APT) of Callus Cultures

Streptomycin, cephalexin and penicillin-G were dissolved in distilled water, filter-sterilized and added to the liquid culture medium after autoclaving. The medium contained all three antibiotics in every case at different concentrations (combination A, B, C – Table 1). Callus cultures (approximately 1 g) were resuspended in 2 ml medium containing antibiotics and incubated for one or two days. In experiment 1 both callus lines (L4 and L2) were used, in experiment 2 line L4 and in experiment 3 line L2 were tested. As control, callus was resuspended in the antibiotic-free liquid medium.

After incubation with antibiotics the medium was filtered off through an 11 μm nylon filter. The cell mass remaining on the filter was washed several times with sterile culture medium to eliminate residual antibiotics. Subsequently, the cell mass was placed onto solid culture medium without antibiotics and cultured for 6 weeks under the growth conditions described above.

The influence of incubation in liquid medium on callus growth was tested in a separate experiment. The growth of callus grown in solid medium throughout the experiment was compared to the growth of callus firstly incubated for 1 or 2 days in liquid medium and then placed onto solid medium for 6 weeks.

The fresh weight (FW) of callus cultures were measured after APT and after 6 weeks of culture and the growth index calculated (final FW – inoculum FW) / inoculum FW).

Table 1. Combinations of antibiotics used for antibiotic pulse treatment of *T. cinerariifolium* callus culture.

| Antibiotic combinations | Concentration (mg/l) | | |
|---|---|---|---|
| | Streptomycin | Cephalexin | Penicillin-G |
| A | 80 | 40 | 2 |
| B | 160 | 80 | 4 |
| C | 320 | 160 | 8 |

### Detecting Bacterial DNA by PCR

PCR analysis of 7 different callus cultures was performed 6 weeks after APT to detect any bacterial contamination. Callus cultures were disrupted in liquid nitrogen and the total DNA isolated and purified as described (Webster & Barker 1994). The polymerase chain reaction using universal eubacterial primers for amplification of 16S rDNA originally designed by Bianciotto & al. 1996 (5' primer 5'-GAG AGT TTG ATC

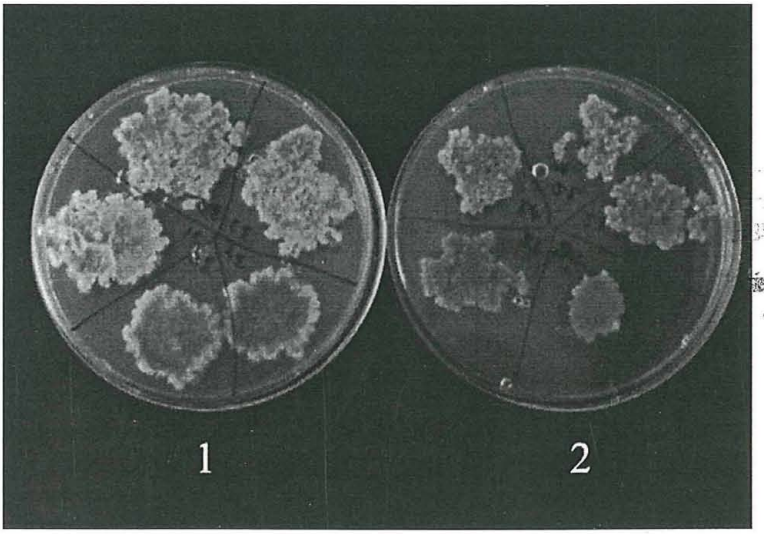


Fig. 1. Callus culture of *T. cinerariifolium* 3 weeks after incubation in liquid medium without antibiotics (1) and with combination B of antibiotics for 2 days (2).

CTG GCT CAG-3', 3' primer 5'-CTA CGG CTA CCT TGT TAC GA-3') was carried out in a Thermocycler GeneAmp PCR System 9700 (Applied Biosystems) as described (BIANCIOTTO & al. 1996). The amplified products (1500 bp) were detected by electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized under UV light.

### Statistical Analysis

ANOVA was used to determine the levels of statistically significant difference (P) between control and callus incubated in liquid medium without antibiotic (Fig. 2), and between control and APT treated callus (Fig. 3). On the other hand, Student t-test was used for the comparison of statistically significant difference between two different pyrethrum genotypes (Fig. 4). Each data point reported in the results is the mean of 5–10 replicates from one experiment $\pm$ standard error (SE). Symbols used in the figures are: *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$; vertical bars indicate SE. The experiments were repeated with reproducible results.

## Results and Discussion

Callus cultures of pyrethrum were initiated using plant material from the Botanical Garden and usually became contaminated after few subcultures. The contamination commonly appeared as a white clouding or veils and was restricted strictly to the medium around the callus (Fig. 1). However, bacteria may remain undetected for long time since the growth medium used for tissue culture may not be optimal for bacteria or may even inhibit their growth (LEIFERT & al. 1994, ISENEGGER & al. 2003). Furthermore, the use of opaque gelling agents such as agar could mask the presence of the contaminant (DEBERGH & VANDERSCHAEGHE 1988).

Callus cultures were incubated in liquid medium containing different concentrations of antibiotics for one or two days (Table 1) to test the effects of APT on callus growth and bacterial elimination. Since the incubation of callus cultures in liquid medium could affect the callus growth we first tested the effects of incubating and shaking callus in liquid culture medium in the absence of antibiotics. The incubation did not significantly affect callus growth (Fig. 2). After one day of treatment the growth index was slightly lower than for callus grown in solid medium. However, after 2 days the growth index was actually higher than for the control.

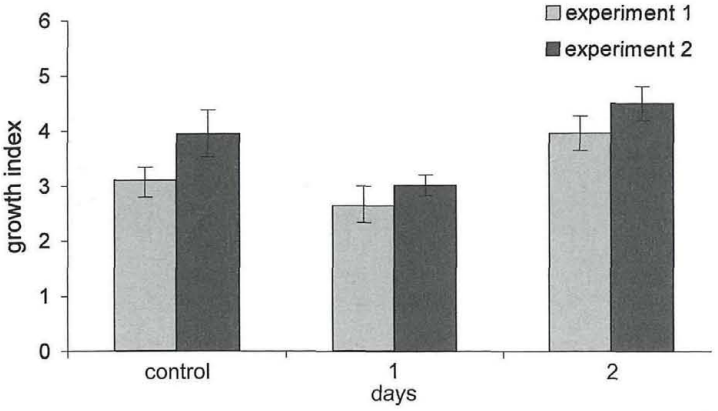


Fig. 2. The average growth index of *T. cinerariifolium* callus cultures 6 weeks after incubation in liquid medium without antibiotics for 1 or 2 days. The control was callus grown in solid medium throughout the experiment. The bars illustrate standard error (±SE).

Previous studies on in vitro cultures of different plant species have shown that streptomycin and penicillin-G can affect growth. The response is usually species-dependent, as in the formation of adventitious roots, which was promoted in *Panax* ginseng (TENG & NICHOLSON 1997). On the other hand, regeneration was inhibited in Platycerium bifurcatum, Clematis, Delphinium, Hosta, Iris and Photinia, particularly when higher concentrations of antibiotics were used (TENG & TENG 2000, LEIFERT & WAITES 1992). Furthermore, in thin cell layer culture systems of tobacco and chrysanthemum, penicillin-G and streptomycin strongly decreased shoot fresh weight (TEIXEIRA DA SILVA & al. 2003).

In our study, the susceptibility of callus cultures to antibiotics was affected by the pyrethrum genotype, duration of APT and the concentrations of antibiotics used in the experiment. After one day of treatment with the antibiotic combination A (80 mg/l streptomycin, 40 mg/l cephalexin, 2 mg/l penicillin-G) no effect on callus growth was observed (data not shown).

After two days of treatment with the same antibiotic combination, callus growth was significantly inhibited (Fig. 3-columns A). Combination

B provoked even stronger suppression (Fig. 3-columns B). However, the growth index was particularly low when 320 mg/l streptomycin, 160 mg/l cephalexin and 8 mg/l penicillin-G (antibiotic combination C) were used (Fig. 3-column C).

Suppression of growth of pyrethrum by antibiotics was also genotype-dependent. After two days of treatment, the growth index differed significantly between the lines L2 and L4 incubated with antibiotic combinations A or B, compared to control (Fig. 4). Treatment with antibiotic com-

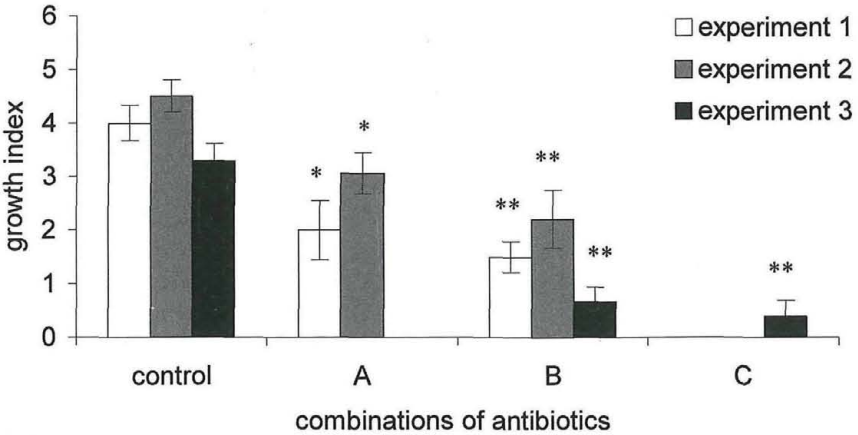


Fig. 3. The average growth index of *T. cinerariifolium* callus cultures 6 weeks after treatment with antibiotic combinations A, B and C for 2 days. The control was callus incubated in liquid medium without antibiotics. The bars represent standard error (±SE). Significant differences between control and treated callus are indicated by asterisks (* $P<0.05$, ** $P<0.01$).

bination A provoked a strong inhibitory effect in line L2, whereas line L4 was only slightly affected (Fig. 4 – columns A). The genotype-dependent difference in response to antibiotics was also observed with concentration B, although it was less pronounced (Fig. 4 – columns B). This observed difference in response to antibiotics between different genotypes is consistent with previous reports on three tansy genotypes (KESKITALO & al. 1998).

The small subunit 16S rDNA sequence was reported to be useful for detecting bacteria in plant tissues (SEAL 1997), especially since many endophytic bacteria cannot be detected by standard microbiological methods. Its application with degenerate universal primers for bacterial detection has been effective and, although species level identification was not achieved, the primers have been useful in detecting a number of bacterial genera (HERMAN 1999). The primers used in our study amplify only 16S rDNA of eubacterial origin, but not mitochondrial or chloroplast DNA of eukaryotic cells, and are thus suitable for detecting bacteria in different tissues (BIANCIOTTO & al. 1996).

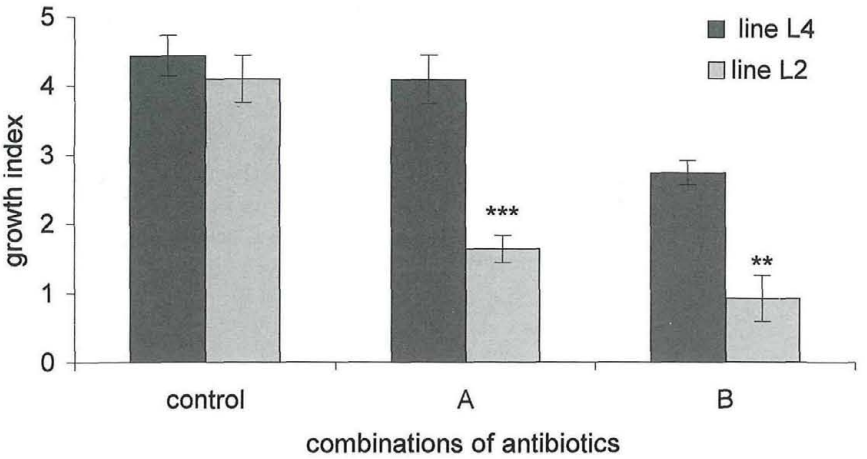


Fig. 4. The effect of genotype on suppression of growth of *T. cinerariifolium*. The average growth index is shown for two different callus lines L2 and L4 6 weeks following treatment with antibiotic combination A or B for 2 days. The control callus was incubated in liquid medium without antibiotics. The bars represent standard error (±SE). Significant differences between the two callus lines are indicated with asterisks (** $P<0.01$, *** $P<0.001$).

In our study, we performed PCR 6 weeks after pulse treatment of the pyrethrum callus cultures. PCR analysis always confirmed the presence of bacteria in visibly contaminated callus cultures. It further showed that there were no bacteria in the callus line L4 incubated for 2 days in medium containing 320 mg/l streptomycin, 160 mg/l cephalexin and 8 mg/l penicillin-G (antibiotic combination C). This line grew further and no visible contamination was observed during several subcultures. In line L2, with the same concentrations of antibiotics, bacteria were not eliminated. Furthermore, bacterial contaminants were also detected in line L4 when lower concentrations of antibiotics (antibiotic combination B) were used (Table 2).

Table 2. The effects of antibiotics on callus contamination and growth of *T. cinerariifolium* 6 weeks after antibiotic pulse treatment for 2 days.

| Callus line | Antibiotic combination | Visible contamination | PCR detection of bacteria |
|---|---|---|---|
| | Control | + | + |
| L2 | B | + | n.t. |
| | C | – | + |
| | Control | + | + |
| L4 | B | + | + |
| | C | – | – |

n.t. not tested

It is interesting that callus treated with antibiotics combination C showed no visible contamination after 6 weeks, although bacterial contamination was detected by PCR analysis in line L2. Thus, visual observation is an unreliable indicator of the absence of contaminants under plant tissue culture conditions, as reported by ISENEGGER & al. 2003.

In conclusion, these data indicate that, although higher concentrations of the antibiotics tested greatly reduced callus growth, they could be used to eliminate bacterial contamination from contaminated pyrethrum callus culture. It is evident that the response and sensitivity to antibiotics is genotype-dependent and therefore an appropriate starting material plays a decisive role in the successful elimination of contaminants. PCR is a rapid and sensitive method for detecting contaminants in pyrethrum callus culture and can offer considerable advantages, particularly in detecting latent and invisible bacteria.

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