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Tissue culture of pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Schultz Bip.)

Tkivna kultura bolhača (Tanacetum cinerariifolium (Trevir.) Schultz Bip.)

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Abstract. Pyrethrum (*Tanacetum cinerariifolium*), is plant species with the highest amount of natural insecticides – pyrethrins. An in-house information system for the development of different plant tissue cultures and marketing of their products was designed. The application of the relational information system in setting up a research hypothesis of high probability is discussed. By processing the information system, plant tissue cultures' parameters were identified, selected and modified. They were tested on the plant tissue culture of *Tanacetum cinerariifolium.* The influence of jasmonic acid on axillary shoot differentiation was studied. An inhibitory effect of jasmonic acid on shoot tissue culture differentiation was proven (100 μM, 10 μM), and a prediction method for determination of the variable optimal concentration interval was presented (between 0,5 and 4,5 μM). In addition a HPLC method was introduced for pyrethrins determination.

Key words: *Tanacetum cinerariifolium*, pyrethrum, plant tissue culture, shoot tissue culture, axillary shoot, natural insecticides, pyrethrins, jasmonic acid, information system

Izvleček. Bolhač (*Tanacetum cinerariifolium*) je rastlinska vrsta z največjo vsebnostjo naravnih insekticidov – piretrinov. Postavili smo informacijski sistem za razvoj različnih sistemov tkivnih kultur in trženje njihovih proizvodov.

Preučevali smo uporabo relacijskega informacijskega sistema za vpeljavo znanstvene hipoteze z visoko verjetnostjo. Z njegovo uporabo smo prepoznali, izbrali in priredili parametre sistema tkivnih kultur. Kot poskusni sistem smo uporabili tkivno kulturo bolhača in ugotavljali vpliv jasmonske kisline (JA) na tvorbo zalistnih poganjkov v tkivni kulturi. Dokazan je bil zaviralni učinek JA na diferencijo kulture poganjkov (100 μM, 10 μM) in predstavljena metoda za napoved intervala spreminjanja optimalne koncentracije JA (med 0,5 in 4,5 μM). Uvedli smo metodo HPLC za ugotavljanje vsebnosti piretrinov.

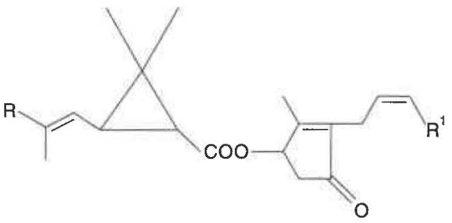
Ključne besede: *Tanacetum cinerariifolium,* bolhač, rastlinska tkivna kultura, tkivna kultura poganjkov, zalistni poganjki, naravni insekticidi, piretrini, jasmonska kislina, informacijski sistem

Abbreviations: MS = Murashige and Skoog growth medium; JA = jasmonic acid; HPLC = high pressure liquid chromatography; IAA = indole-3-acetic acid; 2,4 D = 2,4-diclorophenoxy acetic acid; NAA = naphthalene acetic acid; BAP = 6-benzylamino purine; SD = standard deviation; R = correlation index; EPA = Environmental Protection Agency

Introduction

About 10 000 million insect species cause foodstuff and wood damage. Some species are even fatal, especially for people (ELLIOT 1995). But an extensive usage of synthetic persistent insecticides in the last fifty years has led to their dangerous accumulation in the environment and rapid insect resistance development (COSHRAN 1995). One possible way to minimize the effect of insecticides on biological diversity, and at the same time to ensure food quality, is the application of biodegradable insecticides with low toxicity for Mammals. One of the most promising group of such insecticides are pyrethrins (CASIDA & QUISTAD 1995).

Pyrethrins are natural stereoisomer mixtures of six monoterpenes esters (Fig.1). The basic components of pyrethrins are rethrolone (pyrethrolone, cinerolone and jasmolone) alcohols esterified with chrysanthemic monocarboxylic (pyrethrins I) or dicarboxylic/pyrethric (pyrethrins II) acid (TOMLIN 1994, CROMBIE 1995). Pyrethrins precursor chrysanthemic acid is a volatile monoterpene formed from mevalonic acid of isoprenoid metabolism. Chrysanthemyl alcohol with its typical cyclopropane derives from two molecules of isopentenyl pyrophosphate (IPP). Oxidation of chrysanthemyl alcohol yields chrysanthemic acid. The both have been identified from explant derived callus of pyrethrum. Chrysanthemic acid is found not only as the precursor of chrysanthemic dicarboxylic/pyrethric acid and pyrethrins I/II but also the important precursor in squalene synthesis (KESKITALO 1999).



- pyrethrin I	R	CH3,	R1	CH=CH2
- jasmolin I	R	CH3,	R1	CH2CH3
- cinerin I	R	CH3,	R1	CH3
- pyrethrin II	R	CO2CH3,	R1	CH=CH2
- jasmolin II	R	CO2CH3,	R1	CH2CH3
- cinerin II	R	CO2CH3,	R1	CH3

Figure 1: Monoterpenes esters of pyrethrins.
Slika 1: Monoterpenski estri piretrinov.

The detailed synthesis of rethrolones still remains unclear. However the rethrolones, charecterized by the cyclopentene, are derived most properly from fatty acid metabolism. The composition of the crude extracts of pyrethrum flowers is as follows: one third is the active constituents pyrethrins and two thirds are linoleic and linolenic acids (40 %), alkanes and terpenoides (CROMBIE 1995, KESKITALO 1999). The origin of jasmolone presumably derives from linoleic acid via 12–oxophytodienoic acid (12–oxo PDA). Experimentally is firmly established that 12 oxo–PDA undergoes hydrogenations and ß–oxidations to form jasmonic acid (CROMBIE 1995).

A natural means for controlling a wide range of insects and rapid insecticidal action by pyrethrins is found in the composition of esters mixture. It was also found that a moderate use might cause a slow insect resistance development (Coshran 1995). Pyrethrins were found in some species, e.g. T. coccineum, but more potent is pyrethrum (*T. cinerariifolium*). From the middle of 19th century to World War I, Dalmatia dominated in the pyrethrum production and trade. After the war the domination was replaced by Japan, and after World War II by Kenya, Tanzania and Australia (WAINAINA 1995, GULLICKSON 1995). There were always constraints on ensuring a sufficient supply because of the laborious work, demand for low labour costs, and weather dependency of plant production. In recent years, the efficacy of pyrethrins and the unstable supply, which can no longer meet the world demand (JOVETIĆ & DE GOOIJER 1995, ROYAL SOCIETY OF CHEMISTRY 1988, 1996), stimulated interest for their biotechnological production (HITMI & al. 2000). Analysis of patents concerning pyrethrins in the last thirty-five years has shown a distinctive increasing research interest during the last decade (Fig. 2).

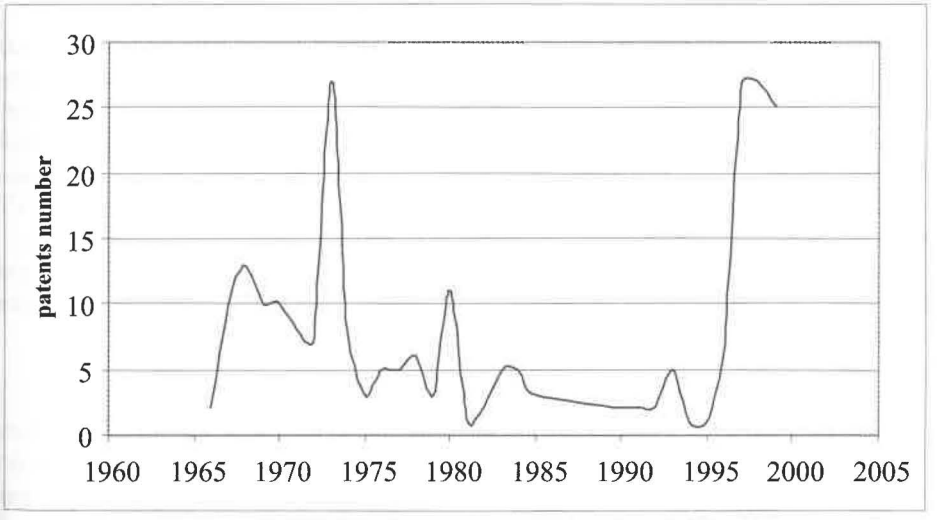


Figure 2: Oscillating pyrethrins research trend in course of time and increasing interest in the last decade (CAplus, march 2000).
Slika 2: Nihanje zanimanja za raziskovanje piretrinov v preteklih letih in naraščanje zanimanja za piretrine v zadnjem desetletju (CAplus, marec 2000).

An alternative production of pyrethrins could be in vitro production by plant tissue cultures, the main advantage of which is flexible control of biotechnological processes. Therefore plant tissue cul-

tures are becoming a tool for producing many important products: e.g. quality food, wood, phytotherapy compounds, biopesticides etc (SASSON 1992, WALTON & al. 1999, RAVNIKAR & ŽEL 1992). The application of the tissue culture technique for the pyrethrum plant has developed also from the self-incompatibility of the plant (PAL & DHAR 1985). For the successful commercialisation of a biotechnological process the most important criterion must be fulfilled – less expensive in vitro in relation to in vivo pyrethrins production (JOVETIĆ & DE GOOIJER 1995).

The basic research problem which we tried to solve was, how to optimise tissue culture cultivation of a commercially potent plant to achieve quality products, e.g. pyrethrins. The problem was structured into several subproblems: (ELLIOT 1995) careful selection of a substance and its source – plant, (COSHRAN 1995) selection of economic cultivating conditions, and (CASIDA & QUISTAD 1995) target-oriented development of cost-effective products. Fragmented information on the defined sub problems is dispersed among several bibliographic and factual sources, while the technological parameters are most often considered proprietary information and are therefore publicly available (HUMPRIES & al. 1991, GULLICKSON 1995). To increase the information content of the accessible and relevant data and information, a relational information system for plant tissue cultures development was designed. The system was tested on the experiment where the effect of JA on shoot production of pyrethrum was studied.

Methods

Tissue culture

Plants of *Tanacetum cinerariifolium* were obtained from the island of Cres, Croatia. Their seeds germinated in vivo and two months old vegetative plantlets of 8 cm height were used in spring for establishment of the shoot tissue culture on MS medium (MURASHIGE & SKOOG 1962) supplemented with 3 % sucrose, 1 % agar, 3 μM IAA and 2 μM BAP The pH was adjusted to 5.7 and the medium was sterilized at 121°C for 20 min. The induced axillary buds had been regulary subcultures, every 4 to 5 weeks, for a year. The tissue cultures were grown in a growth chamber at 23 +/- 1°C under 100 – 110 μM $m^{-2}s^{-1}$ daylight illumination with a 16 h photoperiod.

Axillary buds were separated and cultivated in 50 ml test-tubes on modified MS medium with different concentrations of JA and control medium without JA. In two experiments three JA concentrations were tested: 1 μM, 10 μM and 100 μM JA.

Growth and differentiation measurement

Two and four weeks after subculture (first experiment) the height of the central shoot and shoots number in fifteen test-tubes were determined, and the results were statistically evaluated by Student's t-test: * $P < 0,05$, ** $P < 0,01$, *** $P < 0,001$. In the second experiment the procedure was repeated after three and five weeks.

Differentiation prediction

To fulfil the remaining information gap a prediction method was introduced which will have to be tested in further research. On the basis of all and the selected average shoots number (data inside +/- 2 SD), measured after five weeks of cultivation, the curve with the highest correlation index (R) was determined: at concentration intervals between 0 to 10 μM JA and between 0 to 1 μM JA.

Isolation and quantification of pyrethrins

Approximately 1 g of in vitro grown shoots was crushed in a mortar with a silicic sand and ¼ anhydrous sodium sulfate to obtain a homogenous powder. Pyrethrins were extracted two to three times with 5 ml/g FW petroleum ether (40-60°C). The clear separated extract was evaporated to dryness with rotated evaporator, redissolved in 3 ml CH_3CN, filtered through 0,22 µm mesh filter and analysed.

Pyrethrins were analyzed by Waters HPLC system with a diode array (PDA) detector.

Separations were performed on Nova Pack C18 column (Waters, 150 x 3.9 mm) using a gradient of CH_3CN (solvent A) and Milli Q H_2O (solvent B): 40 % solvent A gradually increased to 80 % during 25 min, followed by a further 10 min at 80 %. Flow rate was 1.4 ml/min. Absorbance was monitored at 225 nm. Pyrethrins in the sample were identified on the basis of retention times and characteristic absorption spectra. Standards used were cinerin I and II, pyrethrin I and II and jasmolin I and II (Pyrethrins technical mixture, PESTANAL, Riedel-de-Haën).

Results and discussion

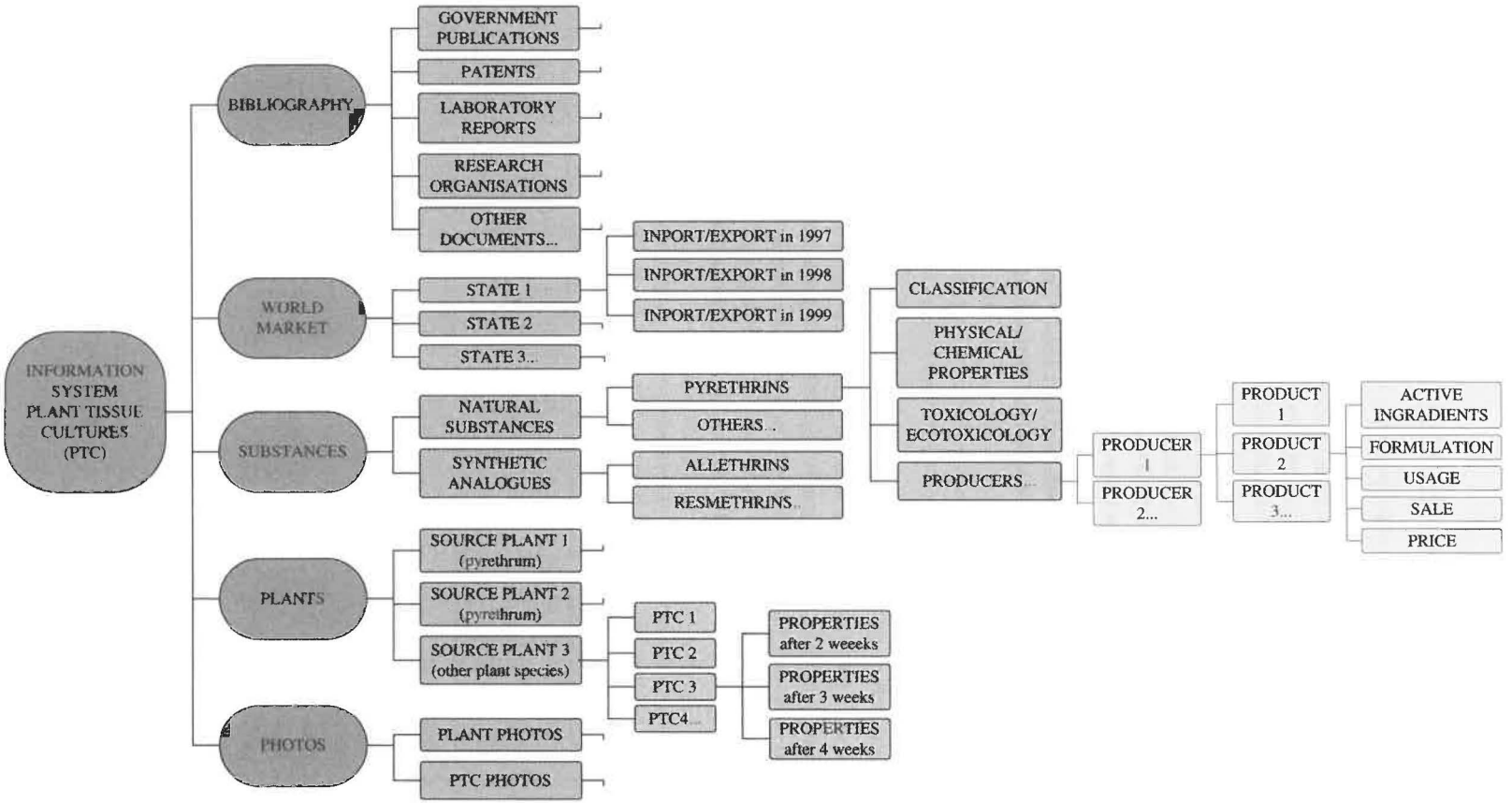
Relational information system

The in-house information system supports problem solving through establishing relationships among five main modules: (a) bibliography, (b) substances (properties, structures, producers, products), (c) world market, (d) plants and (e) photos (Fig. 3). Processing the relational information system for identifying the most effective parameters for biotechnological production of the biosynthetic high-yielding pyrethrum tissue culture clone gave the following result (Table 1):

Table 1: Information densities of breeding parameters' values of pyrethrum plant tissue cultures.
Preglednica 1: Informacijska gostota parametrov vzgoje bolhača v rastlinskih tkivnih kulturah.

Plant tissue culture cultivating parameters	Information densities (number of defined values of specific parameter in documents)
medium modification	38
inoculum	14
basic medium	14
temperature	14
irradiation	13
medium pH	10
plant no.	9
photoperiod	9
light quality	4
inoculum modification	1
storage name	1
storage temperature	1

Figure 3: The structure of five main modules (oval boxes) of the relational information system on plant tissue culture development.
Slika 3: Struktura petih glavnih modulov (ovalna polja) relacijskega informacijskega sistema za razvoj rastlinskih tkivnih kultur.



Based on the information density (Table 1), the basic parameter which promotes plant production is medium modification. This result led to setting up a high probability hypothesis which assumed that the target-oriented medium modification would have the strongest impact on the growth, differentiation and biosynthetic activity of the pyrethrum tissue culture. The information gap on medium modification possibilities was bridged by additional processing of the Bibliographic module of the Relational information system, which gave examples of medium modifications with pyrethrin biosynthesis stimulation effect (Table 2).

In the literature the following pyrethrum tissue culture's stimulation medium modifications were recognised (Table 2): auxins 2,4 D, NAA; cytokinins BA, BAP, kinetin; vitamin ascorbic acid, sucrose, diluted basic medium MS and nutrient stress. It is important to stress that, regarding the bibliographic search in Chemical Abstracts, jasmonic acid (JA) was not reported as a pyrethrum plant growth regulator although it is quite widespread among plants. It is gradually synthesised from linoleic acid, as is suggested for pyrethrins rethrolones (CROMBIE 1995).

Table 2: Medium modifications with pyrethrins biosynthesis stimulative effect obtained from bibliographic module of the information system.
Preglednica 2: Gojišča, ki pospešijo biosintezo piretrinov. Podatki iz bibliografskega modula informacijskega sistema.

Reference	Medium modification
HITMI & al., 1998	2 µM BAP; 21,5 µM NAA
HITMI & al., 1997	2 µM BAP; 21,5 µM NAA; ½ MS;1,8 g/l sucrose
DHAR & PAL, 1993	2,3 µM 2,4 D; 2,2 µM BA
RAJASEKARAN & al., 1991	0,9 µM 2,4 D; 23,2 µM kinetin; MS without agar and nitrates
RAVISHANKAR & al., 1989	0,57 mM ascorbic acid; 0,9 µM 2,4 D; 2,3 µM kinetin
RAJASEKARAN & al., 1990	0,9 µM 2,4 D; 23,2 µM kinetin

Statistically proven inhibitory effect of jasmonic acid

After two and four weeks an inhibitory effect of 100 µM JA on pyrethrum tissue culture growth and differentiation was observed and statistically proven (Figs. 4, 5). JA clearly inhibited number of

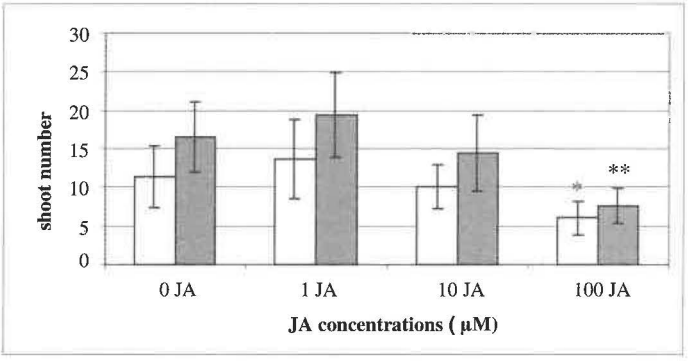


Figure 4: The effect of JA on number of shoots after two (light bar) and four (darker bar) weeks in tissue culture (first experiment). Average shoot number (n>10), standard deviation (SD), and statistical significant differences (t-test) between control media without JA and with different concentrations of JA are shown.
Slika 4: Vpliv JA na število poganjkov po dveh (svetel stolpec) in štirih (temen stolpec) tednih tkivne kulture (prvi poskus). Prikazana so povprečna števila poganjkov (n>10), standardne deviacije (SD) in statistično značilne razlike (t-test) med kontrolnim gojiščem brez JA in z različnimi koncentracijami JA.

shoots, especially on the highest JA concentration (Fig. 4), and the same trend was observed in second experiment (data not shown). JA also inhibited the early lengthening of shoots. After two weeks of culture were shoots on the highest JA concentration significantly smaller then shoots on control media without JA (Fig. 5). Differences were not observed after four weeks of culture.

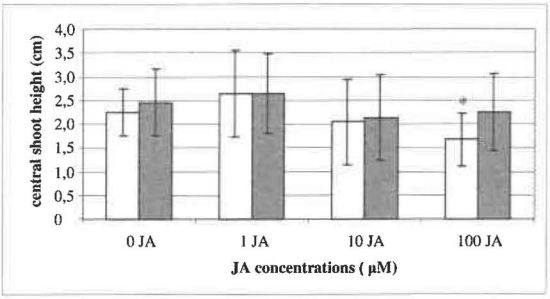


Figure 5: The effect of JA on central shoot length after two (light bar) and four (darker bar) weeks in tissue culture (first experiment). Average shoot number (n>10), standard deviation (SD), and statistical significant differences (t-test) between control media without JA and with different concentrations of JA are shown.
Slika 5: Vpliv JA na velikost poganjkov po dveh (svetel stolpec) in štirih (temen stolpec) tednih tkivne kulture (prvi poskus). Prikazana so povprečna števila poganjkov (n>10), standardne deviacije (SD) in statistično značilne razlike (t-test) med kontrolnim gojiščem brez JA in z različnimi koncentracijami JA.

Prediction of Jasmonic acid stimulative effect

Based on the results (Figs. 4, 5) it was assumed that a stimulatory JA concentration on pyrethrum tissue culture differentiation may lie at the interval between 0 to 10 µM. At intervals between 0 to 10 µM JA and 0 to 1 µM JA the curve that mostly fit both the experimental raw data measured after five weeks of cultivation (second experiment) as well as selected data (+/- 2SD) was found (Figs. 6 and 7). According to the curves´ peaks, which indicate the most copious pyrethrum tissue culture differentiation, the inter-

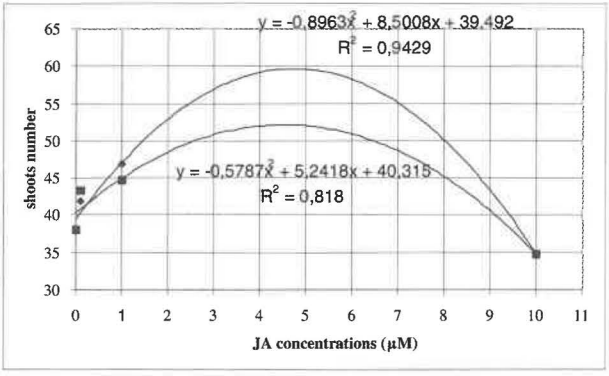


Figure 6: Curves of average values from all (♦) and selected (■) data show at the interval between 0 and 10 µM JA a high dependency (R^2 = 0,9429; R^2 = 0,818) between specific JA concentration and pyrethrum tissue culture differentiation.
Slika 6: Krivulje povprečnih vrednosti vseh (♦) in izbranih (■) podatkov kažejo v intervalu med 0 in 10 µM JA veliko odvisnost (R^2 = 0,9429; R^2 = 0,818) med koncentracijo JA in diferenciacijo bolhača v tkivni kulturi.

val of optimal JA concentrations was determined. The same approach could be used in further research for prediction of other plant tissue cultures properties: e.g. biomass, growth index, pyrethrins content.

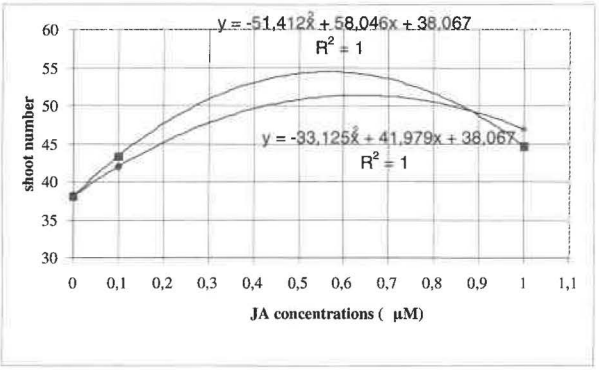


Figure 7: Average values from all (♦) and selected (■) data show at the interval between 0 and 1 µM JA complete dependency ($R^2 = 1$) between specific JA concentration and pyrethrum tissue culture differentiation.
Slika 7: Povprečne vrednosti vseh (♦) in izbranih (■) podatkov kažejo v intervalu med 0 in 1 µM JA popolno odvisnost ($R^2 = 1$) med koncentracijo JA in diferenciacijo bolhača v tkivni kulturi.

Effect of jasmonic acid on pyrethrins content

In all tested cultures six pyrethrins were detected: cinerin I and II, pyrethrin I and II and jasmolin I and II (Fig. 8). Among them pyrethrin I predominated. The total amounts of pyrethrins in the con-

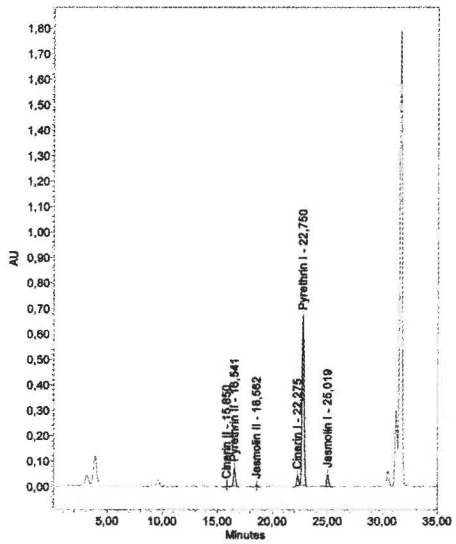


Figure 8: A representative chromatogram: Content of pyrethrins in pyrethrum shoots tissue culture grown four weeks on control medium without JA.
Slika 8: Reprezentativni kromatogram: Vsebnost piretrinov v poganjkih bolhača po štirih tednih tkivne kulture na kontrolnem gojišču brez JA.

trol and JA treated cultures never exceeded 0.44 % of plant dry weight. These were much lower amounts then in flower heads, which contained 0.8 % to 2 % of pyrethrins per dry weight (RAJASEKARAN & al. 1990). The pyrethrins I\II ratio, especially important for insecticide activity, was 0,1 or lower. However in flower heads this ratio is between 0.5 and 3.5 (RAJASEKARAN & al. 1990). In preliminary detections of pyrethrin content in shoots tissue cultures of pyrethrum, jasmoinc acid did not effect pyrethrin contents.

The total amount was lower in tissue culture then in flower heads, grown in vivo. Further work by different inducers is needed to increase the synthesis of pyrethrins in pyrethrum tissue culture.

Conclusions

A systematic selection of economically important pyrethrins produced in tissue culture conditions was outlined. Target-oriented research supported by the in-house information system is a time-effective contribution to the less expensive in vitro pyrethrin production.

The specific inhibitory effect of the chosen entry variable jasmonic acid on *T. cinerariifolium* tissue culture´s differentiation was proven. A method for variable optimal value prediction was presented and applications for further research were indicated.

Povzetek

Bolhač (*Tanacetum cinerariifolium*) je rastlinska vrsta z največjo vsebnostjo naravnih insekticidov – piretrinov, ki imajo vrsto prednosti pred ostalimi insekticidi, kot sta hitra razgradljivost in majhna toksičnost za sesalce (JOVETIĆ & DE GOOIJER 1995). Danes klasična proizvodnja zaostaja za svetovnimi potrebami (GULLICKSON 1995, JOVETIĆ & DE GOOIJER 1995), zato se je obnovilo zanimanje za biotehnološko pridobivanje piretrinov.

V našem delu smo postavili informacijski sistem za razvoj različnih sistemov tkivnih kultur in trženje njihovih proizvodov. Preučevali smo o uporabi relacijskega informacijskega sistema za vpeljavo znanstvene hipoteze z visoko verjetnostjo. Z uporabo informacijskega sistema smo prepoznali, izbrali in priredili parametre sistema tkivnih kulturi.

Ker do sedaj še ni bilo poročil o vplivu jasmonske kisline (JA) na tkivno kulturo bolhača, smo sistem preizkusili na preučevanju vpliva JA na tkivno kulturo poganjkov bolhača. Vzpostavili smo tkivno kulturo bolhača in ugotavljali vpliv JA na tvorbo zalistnih poganjkov v tkivni kulturi. Dokazan je bil zaviralni učinek JA na diferencijo kulture poganjkov (100 μM, 10 μM) in predstavljena metoda za napoved intervala spreminjanja optimalne koncentracije JA (med 0,5 in 4,5 μM).

Uvedli smo tudi metodo HPLC za ugotavljanje vsebnosti piretrinov. V tkivni kulturi poganjkov smo določili vseh šest piretrinov: cinerin I in II, piretrin I in II in jasmolin I in II. Izmed piretrinov najbolj prevladuje piretrin I. Vsebnost piretrinov v poganjkih na kontrolnem gojišču in na gojišču z JA nikoli ne presega 0,44 % suhe mase, kar je veliko manj, kot je piretrinov v cvetnih glavicah, ki vsebujejo od 0,8 % do 2 % piretrinov v suhi masi.

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