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Anti-Fungal Activity and Allelopathic Influence of Vitex agnus-castus L. (Verbenaceae) Essential Oils on Actinidia deliciosa in vitro Culture

N. Ya. Levchyk*, N. V. Skrypchenko, A. V. Liybinska, O. P. Yunosheva, O. I. Dziuba, D. B. Rakhmetov

M. M. Gryshko National Botanical Garden of NAS of Ukraine, Kyiv, UKRAINE
* Corresponding author: levchyk.n@ukr.net

Abstract. The results of research of *Vitex agnus-castus* L. plants from the collection of M. M. Gryshko National Botanical Garden, NAS of Ukraine are given. The species belongs to a group of prospective essential oil plants which are characterized by valuable medicinal, food, aromatic, honey, technical and decorative properties, and can be used in many industries, including pharmaceutical, cosmetic and food. The quantitative content and qualitative composition of the vegetating plant's essential oil are determined, its antifungal influence on the cultures of *Aspergillus niger, Alternaria alternata* and *Fusarium culmorum* is assessed. Antifungal properties of *V. agnus-castus* essential oil were used to sterilize *Actinidia deliciosa* (A. Chev) C.F. Liang & A.R. Ferguson explants introduced *in vitro*. *V. agnus-castus* essential oil is shown to have allelopathic effect on *A. deliciosa* plants. The possibility of using the oil *in vitro* to cultivate *A. deliciosa* callus as a source of bioactive substances and to enhance the effectiveness of biotechnological methods of plant propagation is discussed.

Kay words: *Vitex agnus-castus*, essential oils, allelopathic and antifungal activity, *in vitro,* micro clonal reproduction, callus.

Introduction

Micro propagation technologies are an important complement to traditional plant breeding, ensuring the possibility of achieving large numbers of planting material of unique genotypes, valuable species, and new varieties. Biotechnological approaches, based on tissue and organ culture of plants *in vitro,* provide virus-free plants and solve the problem of multiplying of valuable species and varieties that proved difficulties for conventional breeding methods.

Scientific foundation of biotechnological utilization of plant tissue cultures in pharmacognosy is the ability of cells to synthesize various substances *in vitro*: glycosides, phenolic compounds, cardiac steroids, saponins, lignins, flavonoids, terpenoids, alkaloids, etc. Implementation of cell cultures prevents from extinction thousands of rare plant species capable of synthesizing useful substances. It is known that formation of morphological structures (stems, roots, embrioids) in callus is accompanied by increasing generation of bioactive substances in the culture. Currently, *Ephedra monosperma* C. A. Meyer, *Juniperus sibirica* Burgsd., *Cyclamen kuznetzovii* Kotov & Czernova, and *Cyclamen persicum* Mill. *Paeonia anomala* L. are

successfully cultured *in vitro* in order to produce bioactive compounds in callus (ZARIPOVA *et al.*, 2004; PLYNSKAJA *et al.*, 2008).

Increased production *in vitro* can be achieved by further studies of selection of specialized cell populations and optimization of culture conditions. Main problem is reception of sterile vegetative planting without fungus infection. Thus, there is a search of natural volatile, environmentally safe substances with antifungal and antibacterial properties in order to use them in plant sterilization *in vitro*.

Among similar antibacterial substances of widely distributed plant species there are phenolic compounds of varying complexity: phenolic acids (gallic, caffeic, benzoic, salicylic, etc.), flavonoids, naphthoquinones, and hydrolyzed condensed tannins (LEVCHYK, 2013), the volatile components of essential oils, etc. If skillfully used, phytoncides can be reliable agents of controlling sanitary conditions of the biosphere and of plant protection against diseases and pests (GRODZINSKIJ, 1965; GRODZINSKIJ *et al.*, 1987).

Plant species of the genus *Vitex* L. belong to prospective plants with essential oils, due to their valuable medicinal, nutritive, aromatic, honey, technical and essential oils decorative properties that qualify them for use in various industries such as pharmaceutical, cosmetology and food. The above-ground part of *Vitex* plant appears to contain flavonoid glucoside kasticine, vitexin, and carotene (41.7%), glycoside derivatives of aucubin and p-oxybenzoic acid, iridoid glycosides, essential oils, agnuside, and sytosterol (HOBBS, 1998; HOBERG, 1999; ZASADA & SCHOPMEYER, 2008; STOJKOVIC *et al.*, 2011; DOGAN *et al.*, 2011). Leaves of *Vitex negundo* L. plants also contain iridoid glucosides, carotene, vitamin C, gluco-nonital, benzoic acid, β-sytosterol, and c-glycoside (HOBBS, 1998; HOBERG, 1999). An important feature, characteristic of leaves of *Vitex* plants is nishindine alkaloid $C_{15}H_{31}ON$, flavones, luteolin 7-glucoside, kasticine (VISHWANATHAN, 2010).

Regarding the content of vitamin C, *Vitex* plants belong to vitamin-rich plants. For example, there are at least 88.9-118.1 mg % of vitamin C in leaves of *V. agnus-castus*, and during secondary growth its content reaches maximum values of 615.8±7.1 mg%. Maximum sugar content is 10.1±0.4 % during secondary growth and budding phases. The amounts of proteins in generative and vegetative organs of *V. agnus-castus* plants are high compared to valuable crops, and reach maximum performance of 23.6 ± 0.6 % during fructification. The proteins mostly consist of aspartic, glutamic, alanine, glycine, and proline amino acids. *V. agnus-castus* essential oils which belong to bioactive substances make up in average 0.36-0.48 % of leaves, in accordance with our research – under 0.65 % and 0.47 % of dried seeds (LEVCHYK & RAKHMETOV, 2015).

In our opinion *Vitex* might be the solution for the mentioned above problem of sterility *in vitro*. The study aimed to determine qualitative and quantitative contents of essential oil of *V. agnus-castus,* introduced in right-bank forest-steppe zone of Ukraine, and its allelopathic and antifungal activity in the conditions *in vitro*.

Materials and Methods

The objects of study were essential oils of plants *V. agnus-castus*, introduced in M. Gryshko National Botanical Garden (NBG) of NAS of Ukraine. Quantitative content of essential oil in vegetative mass was analyzed by Clevenger method according to standards (GOST 24027.2-80, 1999), using SIMAX (Czech Republic) tool for volatile oil analysis. The raw materials for essential oil were sampled according to plant vegetation phases.

Analysis of essential oil was performed according to chromatographic methods (CHERNOGOROD & VINOGRADOV, 2006), using Agilent Technologies 6890 chromatograph with mass-spectrometer detector 5973. INNOWAX capillary CG column with inner diameter 0.25 mm and length of 30 m was used.

Allelopathic activity of the essential oil was studied according to (GRODZINSKIJ, 1973), its antifungal properties analyzed by paper-disc method. Tested fungi culture

were represented by phytopathogenic cultures from the collection of Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, *Aspergillus niger* vanTiegh, *Alternaria alternata* (Fr.) Keisser, *Fusarium culmorum* (Sm.) Sacc. Used doses of volatile oil were 2 and 5 µL.

The influence of essential oil on regenerative abilities *in vitro* were studied on the *A. deliciosa* specimens grown in the NBG collection of vine fruit plants. The vegetative reproduction of these plants by traditional breeding methods is difficult and of low efficiency. According to previous studies, shoots of *A. deliciosa* exhibit low coefficient of phytohormone balance and low regeneration abilities, with the maximum percent of rooting explants not more than 10-20% (SKRYPCHENKO *et al.*, 1999).

In order to culture *A. deliciosa,* explants with unopened buds were taken and sterilized using vacuum filter for 1.5-2 min. in 70 % ethanol and 3 min. in 0.05 % sodium thiomersal, then thoroughly washed with distilled water. Then they were planted in the culture medium MS (MURASHIGE & SKOOG, 1963). After budding, young leaves were at once placed into Petri dishes to regenerate. For regeneration, MS culture medium with 3 mg/L benzyl aminopurine, 25 mg/L of vitamins B1 and B6, and 12 mg/L of iron sulfate $Fe_2(SO_4)_3$x 9 H_2O was used. The experiment was conducted on the explants of *A. deliciosa*, cultured. The explants were placed in culture containers with MS medium without addition of *in vitro* hormones (ZAGOSKINA *et al.*, 2009).

Additional sterilization of *A. deliciosa* explants for further propagation in culture *in vitro* with essential oil of *V. agnus-castus* was added to the experimental containers in varying volumes: 1 µL (test №1), 5 µL (test №2) and 10 µL (test №3), to prevent the emergence of dormant fungal infection in the tissues of plants. An experimental container without volatile oil was used as control. Experiments were done in triplicate.

Results and Discussion

It is known that quantitative content of essential oil of *Vitex* plants is species-specific and changes according to vegetation phase, reaching maximum at the height of plant metabolic processes (Table 1). In the course of studies, in 2011-2013 the content of essential oil in *V. agnus-castus* plants growing introduced in right-bank forest-steppe of Ukraine was 0.51%-0.65 %. Optimal period for maximum collecting of oil are the flowering, fructification phases, and the end of vegetation.

Qualitative analysis of essential oil of *V. agnus-castus* shows that its main components are 1,8-cineole, sabinene, limonene and the α-pinene (Table 1). Though the percentage ratio of the components does somewhat change in the course of vegetation, the major components per species remain constant regardless of the plantation's location (LEVCHYK, 2013).

It is noteworthy that 1,8-cineole dominated among oil components in 2012–2013, whereas in 2011 its percentage in the oil was minimum. Qualitative composition of volatile oils is constantly changing, because parallel to the oil secretion and accumulation are the processes of evaporation and losses to the environment.

According to our studies, essential oil of *V. agnus-castus* has potent fungicidal and fungistatic influence on test cultures of *A. niger*, *A. alternata*, *F. culmorum* (Fig. 1). The homeostasis of fungal cells was disturbed, resulting in full inhibition or partial suspension of mycelium growth, lack of sporogony, and changes in pigment generation.

It is found that the tested cultures of *A. niger*, *A. alternata*, *F. culmorum* differ in resistance to toxic influence of *V. agnus-castus* essential oil. The most resistant to this influence is the culture of *A. alternata.* The level of antifungal activity of volatile oil changed in accordance with the vegetation phase, highest during flowering, fruiting and at the end of vegetation, relating to the changes in qualitative composition and quantitative contents of essential oil over the course of plant vegetation. The development of fungal culture of *F. culmorum* was most influenced by essential oil of *V. agnus-castus* produced at the end of plants' vegetation. The oil activity persisted for ten days, and

the aftereffects continued for two months. It was evidenced by the changed color of mycelium of fungal culture from cherry-pink to beige (Fig. 1).

Table 1. Qualitative and quantitative contents of *Vitex agnus-castus* plants' essential oil according to vegetation phases (%), 2013.

Main components	Vegetation phase			
	Second growth – budding	Flowering	Fructification	End of vegetation
1,8-cineole	23.04	23.08	18.38	24.76
sabinene	12.25	13.45	15.10	15.62
limonene	-	6.71	8.28	9.25
a-pinene	9.14	10.19	11.93	10.14
β-caryophyllene	0.84	1.60	1.95	-
caryophyllene-oxide	23.08	2.17	1.55	0.87

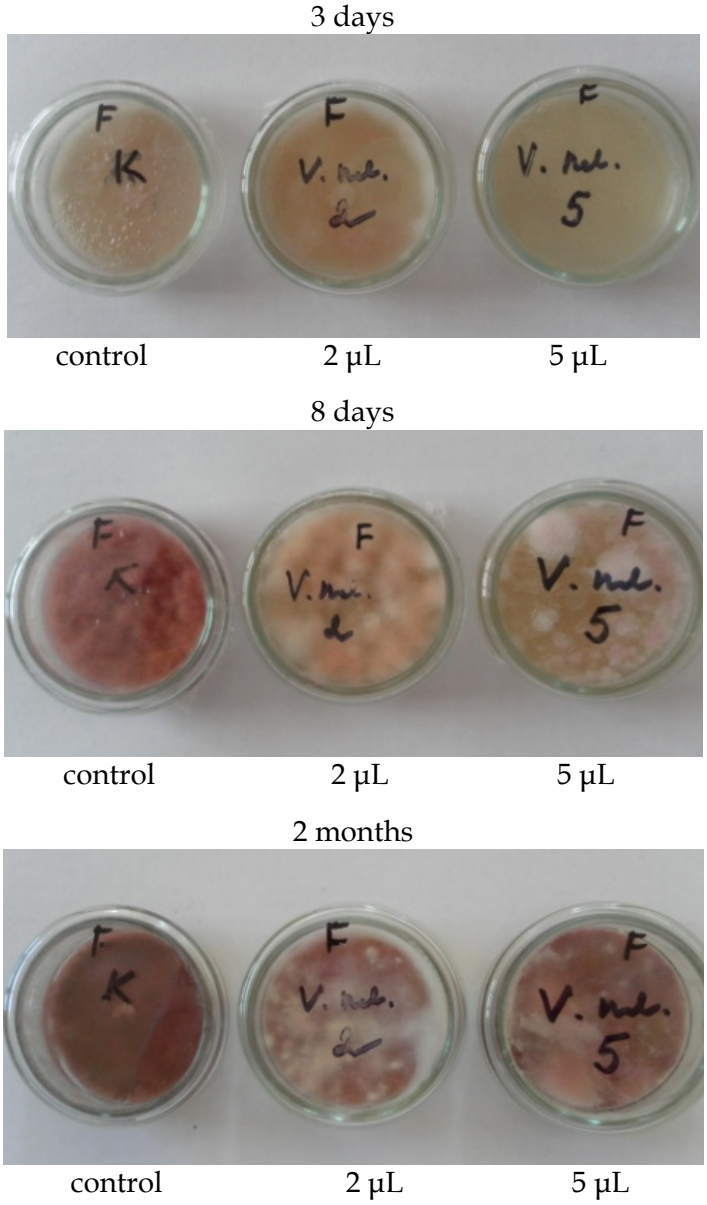

Fig. 1. Influence of essential oil of *V. agnus-castus* on growth and development of *Fusarium culmorum* culture depending on the oil volume (2 µL or 5 µL) and duration of culture (3, 8 days or 2 months) versus control (K).

Table 2. Influence of essential oil of *Vitex agnus-castus* L. on culture growth of *Aspergillus niger* and *Alternaria alternata* (diameter of growth suspension zone, mm) depending on vegetation phase and concentration of volatile components per medium volume.

Phenophase	Oil amounts (µL)	*Aspergillus niger*, exposition, days					*Alternaria alternata*, exposition, days	
		3	6	8	10	Aftereffect	3	6
Secondary growth	2	10	-	-	-	-	-	-
	5	*	-	-	-	-	11	10
Budding	2	12	8	8	8	8	*	-
	5	*	*	8	8	8	10	8
Flowering	2	10	8	9	9.5	9.5	9	7
	5	10	8	8	8.5	6	13	8
Fruiting	2	11.5	9.5	8	7	8	9	8
	5	13	10.5	9	10	10	13	11.5
End of vegetation	2	11	-	-	-	-	10	7
	5	13	10	-	-	-	11	10

Note: * poor development of mycelium; - no GSZ (growth suspension zone).

Antifungal activity of *V. agnus-castus* essential oil against *A. niger* and *A. alternata* manifested in suspension of mycelium growth and sporogony and lower density of fungal culture (Table 2). Although, the influence of essential oil of *V. agnus-castus* on *A. alternata* was in less degree than on other fungal cultures.

Application of essential oil suspends the mycelium growth most effectively if the oil was produced by flowering or fruiting plants. Suspension of sporogony of *A. niger* culture due to influence of essential oil of *V. agnus-castus* is observed on all of the plant's vegetation phases and is directly related to the oil dosage. At the end of plant vegetation, fungal sporogony is shown to be suspended, the GSZ becoming unnoticeable. Suspension of sporogony and culture growth of *A. niger* due to influence of the volatile oil of *Vitex* plants continued for two months, that of the *A. alternata* culture – for ten days.

In order to implement the observed antifungal properties of *V. agnus-castus* volatile oil, it was used for maintenance of sterilization of *A. deliciosa* explants that were cultured *in vitro*. The *in vitro* graftage and direct regeneration were ineffective for these plants, due to the dense pubescence of their leaves and stems, whereas the approach of culturing sterile plants through regeneration showed promise.

After rootless young plants formed on the callus of *A. deliciosa*, they were detached and placed in separate containers with MS medium. In one month, the young plants sprouted roots and developed stem system with sufficient foliage. It should be noted that formation of vast amounts of callus is necessary for rooting of *A. deliciosa* plants in case of micro propagation.

Table 3. Growth characters of *A. deliciosa* plants under allelopathic influence of volatile oils of *V. agnus-castus* plants

Version of test	Callus mass, g	Callus, %	Root (1 month)	Root (2 months)
Control	0.613	100	2.67±0.12	4.50±2.50
1 µL	1.226	200	–	–
5 µL	–	35	–	–
10 µL	–	12-15	–	–

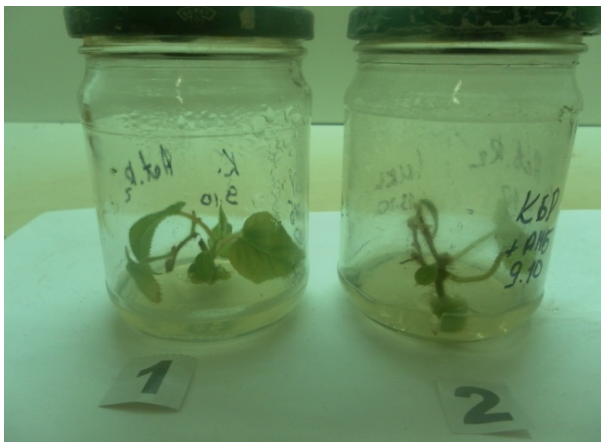

Fig. 2, a. *A. deliciosa* plants
1 month exposition: 1– control;
2 – test №1.

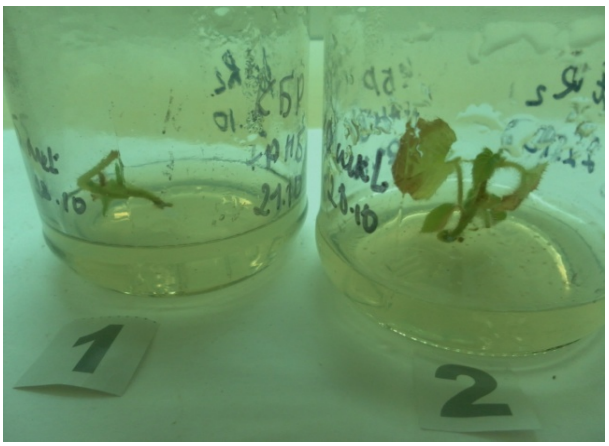

Fig. 2, b. *A. deliciosa* plants
1 month exposition: 1– test №2;
2 – test №3.

Fig. 3. Plants of *A. deliciosa* after two months of experiment (left to right): control, test №1 (1 µL), test № 2 (5 µL), test №3 (10 µL).

Fig. 4. Plants of *A. deliciosa* at the end of experiment
(left to right: control, test №1, test №2, test №3).

For the first month, all explants developed almost identically regardless of the added volume of essential oil of *V. agnus-castus*. After two months of experimentation, significant differences were observed between samples: the control plants developed callus, roots and sufficient leaf mass of vibrant green color. The plants of test №1 (Fig. 2) developed much more callus tissue compared to control, but formed medium-sized leaves and no roots. Plants of test №2 formed insignificant callus and no roots, their leaves were small-sized and of pale green color. No processes of growth or development of explants were registered. Unlike the aforementioned, plants of test №3 started to dry up without exhibiting any signs of growth or development.

As a result of three months of observations, it was found that the control plants developed into fully functional specimens, test №1 plants formed callus two times heavier of the control mass, but no roots, morphometric parameters of test №2 plants remained unchanged, and test №3 plants lack of vital capacity (Table 3, Fig. 3).

Hence, according to our results, the essential oil of plants of *V. agnus-castus* species has high allelopathic activity (both inhibitory and stimulating), the levels of which depend on the qualitative composition of the essential oil, and concentration of volatile components per volume of medium. The essential oil of *V. agnus-castus* showed the inhibitoriest activity on plants of *A. deliciosa* when added at volumes of 5 and 10 µL.

Conclusions

The qualitative and quantitative contents of essential oil of *V. agnus-castus* plants (the oil making up 0.24-0.65 % of plant composition) are determined. The flowering and fruiting plants have the highest accumulation of the volatile oil. Main components of this oil are 1,8-cineole, sabinene, limonene, and α-pinene.

The essential oil of *V. agnus-castus* exhibits high fungicidal and fungistatic effect on test cultures of *Aspergillus niger*, *Alternaria alternata*, and *Fusarium culmorum*. Its activity level depends on the plant's vegetation phase, and concentration of oil per volume. Test culture of *Alternaria alternata* appears to be the most resistant to the volatile oil's toxic effect. There are implications for practical implementation of this property of the plant species for maintaining sterility of micropropagation and culturing plants *in vitro*. The levels and nature of the *V. agnus-castus* essential oil's effect depend on the oil's concentration per volume.

Antifungal properties of *V. agnus-castus* essential oil were successfully used to sterilize *A. deliciosa* explants introduced *in vitro*.

Allelopathic properties of the *V. agnus-castus* volatile oil is observed *in vitro*, demonstrated by its stimulating or inhibitory effects on plants of *A. deliciosa.* The levels of such activity depend on the concentration of volatile substances per volume of medium. Too high or too low concentrations of the oil in the medium are limiting factors that inhibit or stimulate the new formations.

It was shown that the essential oil of *V. agnus-castus* can be implemented *in vitro* in order to produce callus as a source of biologically active substances, and for bolstering the effectiveness of biotechnological methods of cultivation of *A. deliciosa.*

In order to overcome fungus infection *in vitro* the essential volatile oil of *V. agnus-castus* was suggested. This method is ecological, safe for environment, can substitute harmful antibiotics and chemical active materials.

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