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IN VITRO CULTURE AND SOME BIOCHEMICAL CHARACTERISTICS OF *FITTONIA ALBIVENIS* (LINDL. EX VEITCH) BRUMMITT

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In this work we described a procedure of *Fittonia albivenis* (Lindl. ex Veitch) Brummitt *in vitro* conservation and microclonal propagation by enhanced bud proliferation from shoot tips as well as studying some biochemical characteristics of plants cultured both *in vivo* and *in vitro*. Multiple shoot formation from apical and axillary buds started in 4 months on MS/2 medium without growth regulators. Multiplication coefficients ranged from 6.0 ±1.9 to 14.7 ±2.6 for different plant clones. So, as a result of our work, the effective system of microclonal propagation of *F. albiviens* on hormone-free media was elaborated. It have been shown that application of plant growth regulators seems just to shorten the time period of *Fittonia in vitro* microclonal propagation but does not have any significant effect on multiplication coefficients. The total content of bioactive substances (flavonoids and phenolics) was about 0.2–0.45% of dry weight, that is significantly less than its amount in known sources of polyphenols (e.g., ground coffee). However, the data indicate the significant differences in the content of these bioactive substances between *in vivo* growing plants with dark-green (clone #1) and light-green leaves (clone #2) as well as different reactions to the transfer to the *in vitro* conditions. For some clones the significant increase in both phenolics and flavonoids amount was observed after such transfer. The clones with highest productivity have been identified and will be used in further researches.

Keywords: *Fittonia albivenis*, *in vitro* propagation, phenolics, flavonoids

Introduction

Acanthaceae family consists of 4,300 species in 346 genera and is, therefore, one of the most diverse families of flowering plants. Besides the fact that Acanthaceae plants possess an important ecological role in their native habitats they contain many important secondary metabolites and are used for treatment of many diseases. For example, some of them have the ability to kill or retard the growth of many infectious microbes including Pseudomonas

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species (Shinwari et al., 2017); show antiplasmodial, antitumor and antioxidant activities (Charoenchai et al., 2010), etc.

Fittonia albivenis (Lindl. ex Veitch) Brummitt (synonyms *F. argyroneura* Coem., *F. verschaffeltii* (Lem.) Van Houtte) is a species of Acanthaceae family native to the Peruvian rainforests and known as 'nerve plant' or 'mosaic plant'. According to leaf coloration, *Fittonia* plants are separated into two groups: white-veined plants belong to the *Argyroneura* group while red or pink-veined plants belong to the Verschaffeltii group. Available literature reports several directions of *Fittonia's* practical application. Due to its attractive foliage it is often used as ornamental plant. Leaf venation characteristics make *Fittonia* an object for studying leaf anatomy and physiology (Wook et al., 2012; Bercu and Popoviciu, 2015). Several publications point to *Fittonia's* ethnopharmacological properties. It is reported that aerial parts of *F. albivenis* in combination with *Tabernaemontana sananho* Ruiz and Pav. are efficient for treatment of snakebite wounds (Sanz-Biset et al., 2009; Félix-Silva et al., 2017). *Fittonia* aerial parts are also used in Peruvian Amazon as antidiarrheal for children (Sanz-Biset et al., 2009). Whole plant infusions or decoctions were used as a rinse of the mouth and for toothache therapy (Quattrocchi, 2012). It has been reported that indoor air is polluted with volatile organic compounds (VOCs) that can cause some acute and chronic diseases. An alternative way to reduce the level of VOCs in indoor air is the use of plants. Among indoor plants screened for their ability to remove important VOCs with differing chemistries *F. albivenis* was identified as a species with superior benzene, toluene, and trichloroethylene removal efficiency (Yang et al., 2009; Dela Cruz et al., 2014).

All these data make *Fittonia albivenis* an interesting object for further studies. However, its growing *ex situ* apart from the natural habitat in rainforests requires special conditions and makes it necessary to elaborate efficient methods of cultivation and biomass accumulation for the research activities and practical application. *In vitro* culture can be a promising approach in this respect. The role of biotechnological methods in plant biodiversity conservation and practical application is steadily growing but available *ex situ* plant depositories focus mainly on agricultural crops while there appear to be no major depositories of the medicinal and other wild-growing plants (Cordell and Corvald, 2005). In our research project concerning formation and maintaining *ex situ*/*in vitro* bank of plants representing different taxonomic groups of the world flora the *ex situ* bank contains seed samples of 31 monocotyledonous and 134 dicotyledonous plant families. Aseptic plants, cell lines and embryogenic cultures of 18 monocotyledonous and 92 dicotyledonous families form *in vitro* bank (Belokurova and Kuchuk, 2014). Acanthaceae family is represented in seed bank by some species of *Acanthus*, *Indoneesiella*, *Ruellia* and *Crossandra* genera while in *in vitro* bank *Crossandra nilotica* Oliv., *Indoneesiella echioides* (L.) Sreem., *Ruellia humilis* Nutt., *Ruellia strepens* L. are cultured.

Publications concerning *Fittonia in vitro* cultivation are not numerous. They focus on searching basic principles of tissue culture for this species and studying chimeric stability with different proliferation methods (Chen and Li, 1987; Yang, 2000; Pan et al., 2005; Wang et al., 2015a, b). In addition, information about bioactive compounds synthesized by *Fittonia* plants is very scarce.

The aim of this research was to enrich *in vitro* bank with one more (*Fittonia albivenis*), to elaborate efficient protocols of its *in vitro* multiplication, and to determine the content of some bioactive substances in soil-growing initial plants and aseptically cultured lines. The work is being carried out within the research project 'Germplasm bank of the world flora' (Belokurova and Kuchuk, 2014).

Material and methodology

Plant material

Eleven clones of *F. albivenis* soil-growing plants were used as initial material for studies. These plants were purchased on the local market and grown in the green-house of the Institute of Cell Biology and Genetic Engineering. Aseptic plants obtained during this study were cultivated in the thermal rooms of Germplasm bank of world flora of Institute of Cell Biology and Genetic Engineering. The clones differed with leaf coloration (dark or light green with white, or red, or rose veins of the various value of color) and growth rates in the soil.

In vitro culture and microclonal propagation

Stem fragments with apical and axillary buds of soil-growing plants (five explants of each plant clone) were carefully washed in stream water and used to start *in vitro* culture. Surface sterilization has been carried out using 70% ethanol (30 seconds), then with commercial bleach (resulted concentration of sodium hypochlorite – 0.5%) for 10 minutes. The treated buds were then thrice washed with sterile distilled water, dried on sterile filter paper and placed for cultivation on MS culture medium (Murashige and Skoog, 1962) with twice reduced macro-components and sucrose (medium MS/2). Plant material was cultured at 25 °C and 16-hour-photoperiod with subcultivations to the fresh media every 4 weeks. Growing shoots were cut into pieces containing an axillary bud and subcultured separately. Propagation rates were estimated in 2 months after the beginning of multiple shoot formation (6 months after starting *in vitro* culture) as a number of shoots formed in each cluster. Twenty clusters of each plant clone were used for calculations of statistical confidence.

Determination of the total phenolic content

The Folin-Ciocalteu method was used (Singleton and Rossi, 1965). The method is based on the reaction of compounds having a phenolic group with a Folin-Ciocalteu reagent to form blue-colored complexes whose optical density is measured at a wavelength of 760–765 nm. 200 µl of 10% aqueous Folin-Ciocalteu reagent was added to 100 µl of *Fittonia* ethanol extracts and the obtained solutions were stirred for 20–30 seconds. Then 800 µl of 7.5% aqueous Na_2CO_3 was added and the resulting solutions were left for 2 hours at room temperature; after that the optical density was measured. Ferulic acid was used as a standard substance so phenolic content was expressed as milligram of ferulic acid per gram of dry mass.

Determination of the total flavonoids content

The total flavonoids content was determined spectrophotometrically by measuring the optical density of red-colored complexes formed due to the reaction of flavonoids with aluminum

chloride (Pękal and Pyrzynska, 2014). $NaNO_2$ (360 µl of 5% solution) was added to 1 ml of ethanol extracts and left to incubate for 5 minutes following by adding 600 µl of 2% $AlCl_3$. The solutions were carefully stirred and left for 6 minutes. Then 600 µl of 1 M NaOH was added and incubated for 10 minutes. The optical density of formed red solutions was measured at a wavelength of 510 nm. Rutin was used as a standard substance so flavonoids content was expressed as milligram of rutin per gram of dry mass.

Statistics

Statistical processing of the results was performed using descriptive statistics. In the description of the content of phenolics and flavonoids, the values are presented in the form M ± m, where M is the mean, m is the standart error oth the mean; in the description of the *in vitro* multiplication coefficient m is the standard deviation.

Results and discussion

In our work on the maintenance and study of *in vitro* bank of the world flora we deal in most cases with a restricted quantity of initial plant material for starting *in vitro* culture. As a rule seed samples are used as starting explants but apical and axillary buds can be used as well if the living plants are available. Using shoot tips alternatively to seeds is preferential to maintain clone stability. In this work despite the little quantity of available *Fittonia* buds used as initial material the procedure of surface sterilization was quite successful. Surface sterilization efficiency reached 60–100% for nine of eleven plant clones that allowed obtaining 3–5 aseptic buds of each clone for further induction and growing of shoots. It is sufficient quantity to induce aseptic plants to be multiplied in *in vitro* bank for species conservation.

Hormone-free culture medium MS/2 was used to induce bud development and formation of *Fittonia* shoots as a common procedure for many other species maintained in our *in vitro* collection. Each aseptic bud formed slowly growing single shoot with 4–6 leaves within a period of approximately 3 months. The shoots were divided into the internodes with axillary buds and cultured on the same medium. Their growth was very slow and further development delayed while the basal parts of the stem were gradually enlarging along with some local necrosis of the tissues. Formation of multiple shoot clusters started in 4–5 months in all studied plant clones. Single shoots were cut from the clusters and transferred for rooting to the fresh culture medium of the same composition. Rooting took place in 1.0–1.5 months with the efficiency 90–95%.

Table 1 demonstrates the propagation efficiency of the studied *Fittonia* clones. In general they did not significantly differ according to their multiplication rates although the mean values of the coefficients in some cases were quite far from each other. At the same time significant differences in mean values were detected for clone #2 and #3; #2 and #4; #2 and #10; #2 and #11; clone #3 and #9. Plants #3 showed the lowest multiplication coefficients and growth rate. It should be mentioned that the rates of *in vitro* shoot multiplication of each plant clone in general corresponded to their growth rates in the soil (clones #2 and #9 were the most rapidly growing while clones #3 and #10 were very slowly growing in the soil).

Regular transfer of shoot clusters to the fresh MS/2 medium resulted in increasing multiplication efficiency. As an example, the total quantity of regenerated plants of the most rapidly growing clone #2 per 8-months cultivation reached more than 1,500 plants ready to be transferred to the soil. After 14-months cultivation the propagation rates decreased, and plants were maintained *in vitro* by routine subculturing of shoot tips.

Table 1 Some characteristics of the studied *Fittonia albivenis* (Lindl. ex Veitch) Brummitt clones *in vitro*

Clone	Leaf coloration	*In vitro* multiplication coefficient	Total phenolic content1	Total flavonoids content2
#1	Dark-green leaves and dark-red veins	9.6 ± 4.4	3.14 ±0.21	2.26 ±0.21
#2	Light-green leaves and white veins	14.7 ±2.6	3.88 ±0.30	2.07 ±0.11
#3	Dark-green leaves and rose veins	6.0 ±1.9	not studied	not studied
#4	Green leaves and crimson veins	9.6 ±2.4	2.60 ±0.62	2.13 ±0.20
#6	Dark-green leaves and light-rose veins	10.2 ±3.8	4.32 ±0.16	3.14 ±0.8
#8	Almost entirely pink	9.5 ±3.8	2.78 ±0.53	4.00 ±1.08
#9	Almost entirely dark-pink	11.5 ±3.4	3.70 ±0.80	3.33 ±0.35
#10	Bright-green leaves and crimson veins	7.2 ±2.6	3.97 ±0.43	2.96 ±0.30
#11	Dark-green leaves and dark-rose veins	8.3 ±3.4	3.41 ±0.56	3.02 ±0.37

Notes: 1 – expressed as mg ferulic acid/g of dry weight; 2 – expressed as mg rutin/g of dry weight

There are few publications concerning *in vitro* culture of *F. albivenis* focusing on elaboration of efficient methods of surface sterilization and mass propagation of this species (Chen et al., 1987; Yang, 2000; Pan et al., 2005; Wang et al., 2015) as well as studying chimeric stability with using different proliferation methods (Wang et al., 2015). In the earlier studies (Chen et al., 1987) different combinations of plant growth regulators (6-benzylaminopurine (BA), 1-Naphthaleneacetic acid (NAA), 2.4-Dichlorophenoxyacetic acid (2.4-D), and Indole-3-butyric acid (IBA) were used to study *F. albivenis in vitro* morphogenesis. In other work shoot clusters were induced on MS culture medium supplied with BA and NAA. BA concentrations ranged from 0.1 to 2.0 mg/l while studied NAA concentrations were from 0 to 1.0 mg/l. The highest multiplication coefficient (5.6 during 1-month cultivation) was observed on 1 mg/l BA, and it's increasing up to 2.0 mg/l did not induce higher multiplication rates and even lowered it (Yang, 2000). Pan et al. (2005) have reported that the effect of clump-germ induction from shoot tips was the best on the medium MS + 0.1 mg/l BA + 0.1 mg/l IBA.

In our research, a hormone-free medium was used for growing *F. albivenis* plants to ensure their clonal stability *in vitro* and to minimize any possible morphological and/or other types of variations. As our main aim was *in vitro* conservation the multiple shoot formation can be estimated as some type of "side effect" in the course of cultivation without exogenous plant growth regulators. The rates of multiplication were quite high as those on culture media supplied with growth regulators reported in the literature (Yang, 2000). Comparison of our

results and the other published data shows that application of plant growth regulators seems just to shorten the time period of *Fittonia in vitro* microclonal propagation but does not have any significant effect on multiplication coefficients.

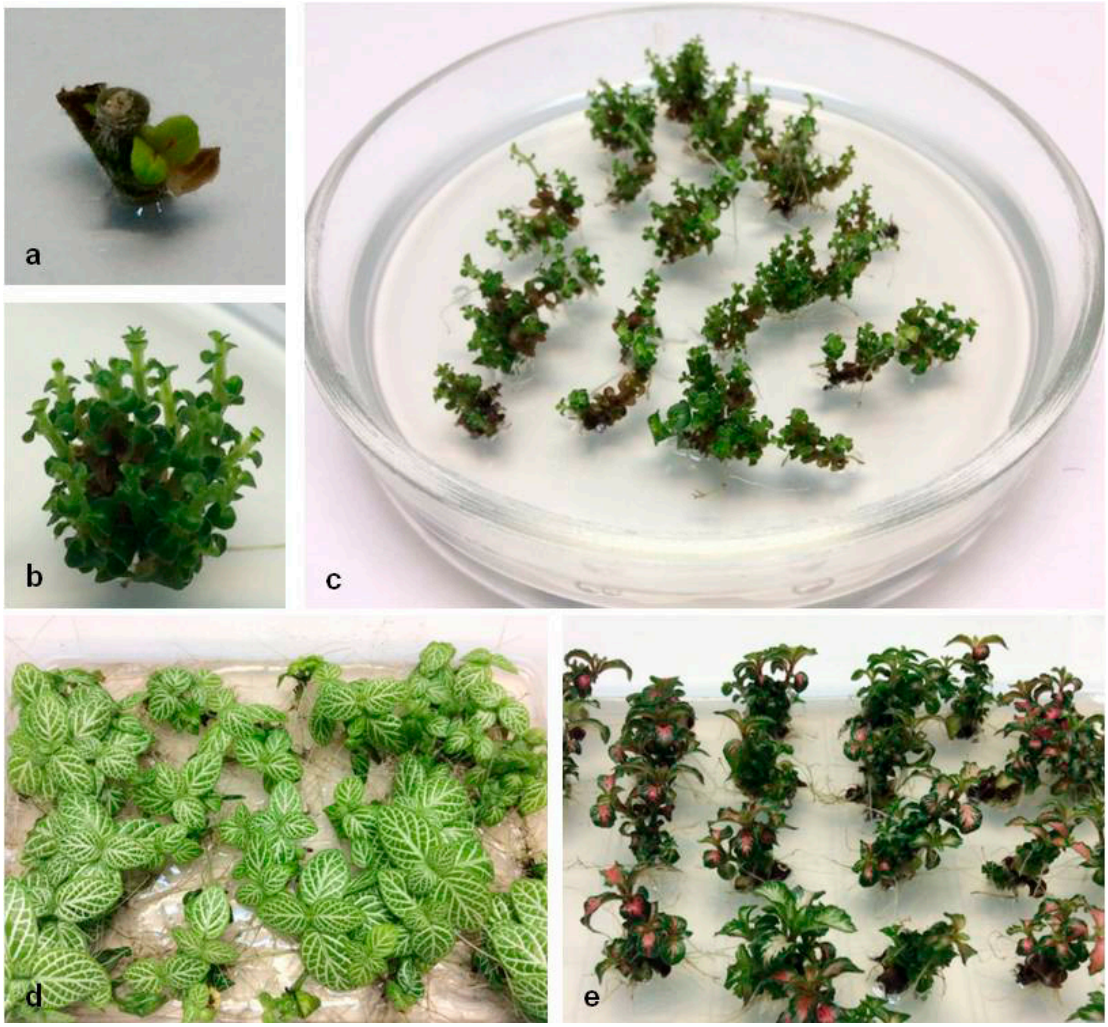

Figure 1 *Fittonia albivenis* multiple shoot formation on hormone-free MS/2 medium
a – a stem fragment with axillary bud used as initial explant; b and c – formation of shoot clusters; d and e – *in vitro* rooted plants of the clones #2 (d) and #9 (e) ready to be transferred to the soil

The results of the determination of total phenolics and flavonoids content are shown in Table 1 and Figure 2 and 3.

The total content of flavonoids and phenolics was about 2.0–4.5 mg (of rutin or ferulic acid, accordingly) on 1 g of the dry weight of plant (0.2–0.45% of dry weight). It is about 10–50 times less than these bioactive substances amount in known sources of polyphenols – e.g., total polyphenol content in ground coffee is about 52–57 mg gallic acid/g dry weight (Ignat et al., 2011). However, the data indicate the significant differences in the content of these bioactive

substances between plants with dark-green and light-green leaves which were cultivated *in vivo*. The dark-green plants (clone #1 *in vivo*) accumulate more phenolic substances and, especially, flavonoids than that with light-green leaves (clone #2 *in vivo*). These clones have also different reactions to the cultivation *in vitro*. In such conditions the content of the investigated substances did not change in dark-green leaves clone (clone #1 *in vitro*) whereas the significant increase in both phenolics and flavonoids amount was observed for light-green leaves one (clone #2 *in vitro*).

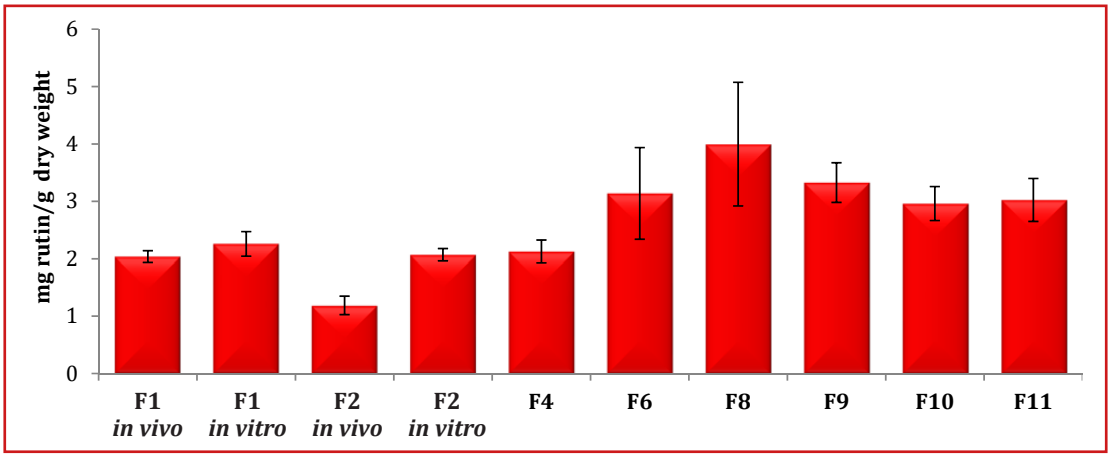

Figure 2 Total flavonoids content of *Fittonia* extracts of *in vitro* and *in vivo* cultivated plants (mean ± standart error of the mean)

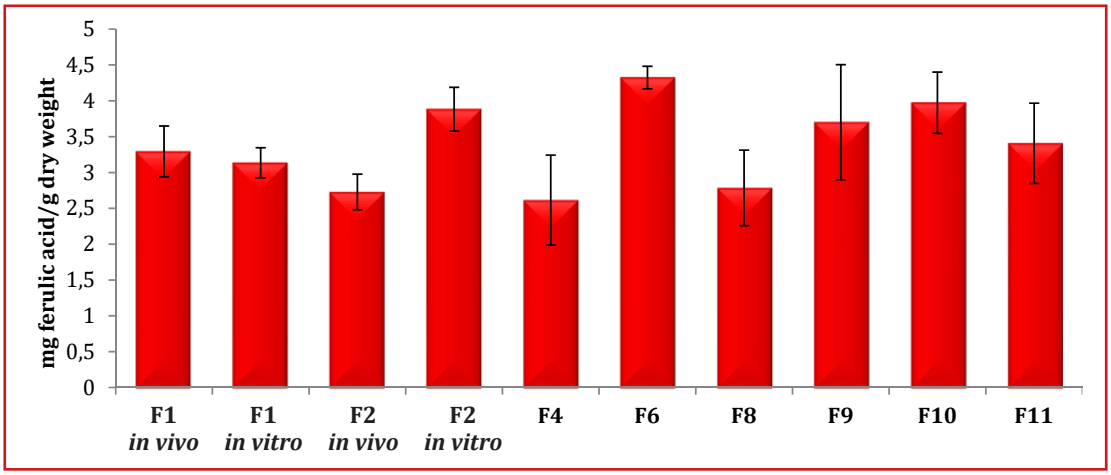

Figure 3 Total phenolics content of *Fittonia* extracts of *in vitro* and *in vivo* cultivated plants (mean ± standart error of the mean)

There is also a significant difference in the amount of flavonoids between different *Fittonia* clones cultivated under *in vitro* conditions. E.g., the flavonoids content was the largest for the clone #8 and it was twice as large as its amount in most of the studied clones (clones #1–4,

11). The amount of phenolic compounds in investigated clones differed to a lesser extent. It should also be noted that there was no significant correlation between the amount of studied bioactive compounds and the coloration of the leaves and/or their veins of the investigated plants.

Conclusions

As a result of our work, the effective system of microclonal propagation of *F. albiviens* on hormone-free media was elaborated. The rates of multiplication were quite high as those on culture media supplied with growth regulators reported in the literature (Yang and Zheng, 2000). It have been shown that application of plant growth regulators seems just to shorten the time period of *Fittonia in vitro* microclonal propagation but does not have any significant effect on multiplication coefficients. The total content of bioactive substances (flavonoids and phenolics) was about 0.2–0.45% of dry weight, that is significantly less than its amount in known sources of polyphenols (e.g., ground coffee). However, the data indicate the significant differences in the content of these bioactive substances between investigated clones as well as different reactions to the transfer to the *in vitro* conditions. For some clones the significant increase in both phenolics and flavonoids amount was observed after such transfer. The clones with highest productivity have been identified and will be used in further researches.

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