IN VITRO REACTIVITY OF *USNEA BARBATA* (L.) MOTT.

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The investigations presented in this paper focused on *Usnea barbata* (L.) Mott. *in vitro* reactivity. Different variants of basal media BBM (1965) and Honegger (1993), have been used. The effects of nutrient media composition on this lichen species *in vitro* culture have been studied. A lower growth rate was registered in both cases on the basal medium with no additives. The presence of kinetin in a concentration of 0.1 mg/l stimulated the induction of proliferation. For the initiation of the *in vitro* cultures two kinds of explants were used. The best results were obtained in the case of the grinding paste inoculi. The results we have obtained determine us to say that *Usnea barbata* (L.) Mott. proved to be highly responsive to the *in vitro* culture and it could be used as an experimental system for secondary metabolites biosynthesis.

Key words: lichens, *in vitro* culture, *Usnea barbata.*

INTRODUCTION

One of the major preoccupations for the biotechnological researches started to be using lichens as a suitable model system in secondary metabolites production by culturing them *in vitro* (Yamamoto *et al.*, 1993; Carmen Molina and Crespo, 2000).

Considering the available scientific literature, we can observe that in numerous studies their pharmacological properties are mentioned (Huneck, 1981; Grigorescu *et al.*, 1986).

Thus, many lichen species, like *Usnea hirta*, *U. barbata*, *U. florida*, *U. longissima* and *U. dasypaga*, are used for medicinal purpose.

Usnic acid confers to these lichens a bitter taste and acts, also, like an antibiotic (www. holisticonline.com).

Usnic acid is a favorable substance for a series of diseases induced by *Staphylococcus*, *Streptococcus* and *Pneumococcus*, *Candida* infections, respiratory and urinary tract affections, indigestion and also, it was successfully used in the treatment of different kinds of cancer.

Comparing usnic acid by rate (%) from the natural thalli and from that *in vitro* cultured (Yamamoto *et al.*, 1993), it has been found out that natural thalli contain a bigger amount (0.90%) compared to that one grown *in vitro* conditions

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(0.16 %); in spite of these, the growth rate in tissue cultures is higher (Dobrescu *et al.*, 1993).

So, lichens culture productivity is bigger as against that of natural thalli, making in this way the cultures potential valid for industrial production. In tissue cultures the usnic acid was detected in the composition of both bionts. These denote that usnic acid production is based on a mycobiont-phycobiont relation (Lawrey, 1984).

Considering that, we have appreciated that lichens introduction *in vitro* culture is a future challenge.

MATERIAL AND METHODS

The selected biologic material was represented by *Usnea barbata* (L.) Mott. species (Fig. 1).

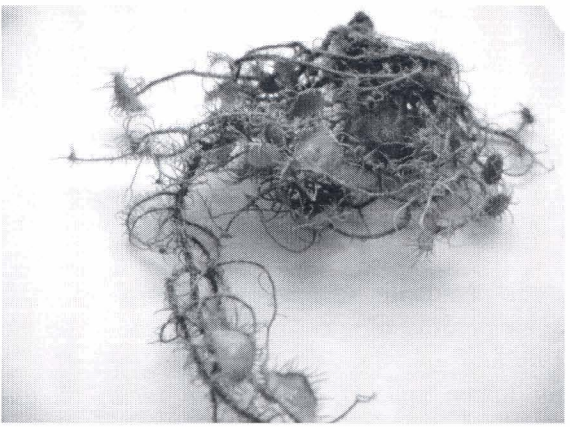

Fig. 1. *Usnea barbata* L. (Mott.) native thallus.

Fresh thalli of *Usnea barbata* (L.) Mott. were collected from resinous tree bark (spruce fir, fir, pine) and deciduous (beech, sycamore maple tree) in mountain regions. The protocols included in the book of Kranner and coworkers (2002) represented a reference point for our experiments.

To introduce this species *in vitro*, three stages were done: sterilization, inoculation and incubation.

For removing a superficial contamination, the biological material was initially washed in water stream for two hours, then in distilled water.

Following this step we have washed the biological material with ethyl alcohol (70 %) for a few seconds, then in dichloroisocyanuric acid sodium salt – 0.5 g/100 ml (in two rounds of 3 minutes followed by washing with distilled water (Cogalniceanu and Stoiculescu, 2007).

The inoculum was represented by thallus fragments and a suspension of grinding thallus, in nutrient media. Both liquid and solidified with gerlite media were parallelly tested. Before inoculation this suspension was filtered twice using a filter with diameter of pores of 140 μm.

The following basal culture media and modified formulae were used:

– Bold's basal medium (BBM), recommended in literature (Kranner *et al.*, 2002);

– Honegger (1993);

– Murashige and Skoog (1962).

To these we have added hormones (cytokinines, kinetin respectively) and another compound such as peptone and glucose (Tables 1 and 2).

A quantity of 1 ml suspension (filtrate) and thallus fragments of 2 mm were inoculated in 2 ml of liquid medium. Periodically, the medium was refreshed by adding fresh medium.

Table 1

The lichen grinding suspension inoculum reactivity to the culture medium

Basal medium	Phytohormones	Additives (g/l)		Reactivity
	kinetin (mg/l)	Peptone	Glucose	
BBM (1965) liquid state	0.1	–	–	+ + +
BBM (1965) liquid state	–	2	4	+
Honegger (1993) liquid state	0.1	–	–	+

Table 2

The thallus fragments inoculum of lichen reactivity to the culture medium

Basal medium	Phytohormones	Additives (g/l)		Reactivity
	kinetin (mg/l)	Peptone	Glucose	
MS (1962) solid state	–	–	–	–
MS (1962) liquid state	–	–	–	–
BM (1965) solid state	–	–	–	–
BBM (1965) liquid state	–	–	–	–
BBM (1965) liquid state	0.1	–	–	++
BBM (1965) liquid state	–	2	4	+
Honegger (1993) liquid state	0.1		–	+

Legend: "–" = non reactivity, "+" = slow reactivity, "+ +" = good reactivity, "+ + +" = very good reactivity

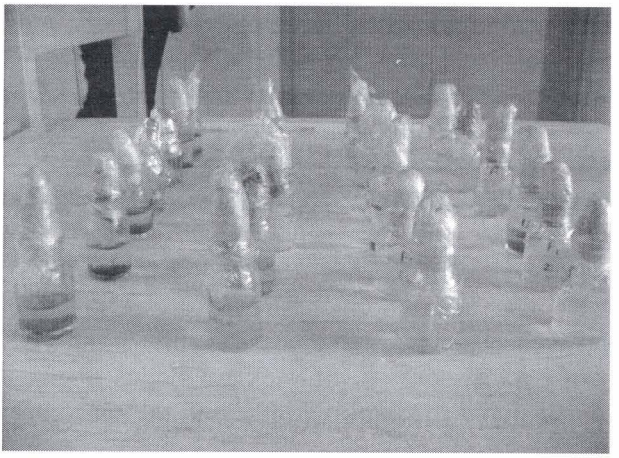

Fig. 2. The *in vitro* cultures in the growth chamber (general view).

Lichen cultures were grown *in vitro* at 24 °C ± 2 °C, at 3000 lux illumination and a photoperiod of 16/8 hours. The cultures were daily observed. Three weeks after, several series of microscopic observations were completed on squashed slides stained with bleu – cotton and using a photonic microscope (©Nikon Eclipse E 200).

RESULTS AND DISCUSSION

Studies concerning the lichen tissue culture experiments have been realized during the past ten years by some scientists (Carmen Molina, 2000; Stenroos, 2003). Yamamoto and coworkers (1993), for example, developed a method to initiate lichen tissue culture from vegetative thalli. Authors have studied the effects of culture conditions on the growth of tissue isolated from three fruticose genera, *Alectoria*, *Ramalina* and *Usnea*. It was observed that in general lichen tissue culture grows much faster compared to the natural thalli and their growth rates may be improved if in the scope of lichen tissue cultures it is targeted the secondary metabolites production. For this purpose, different kinds of nutrient media as well as experimental conditions (light, temperature, etc.) were analysed.

Our experiments revealed that the reactivity of *Usnea barbata* (L.) Mott. in *in vitro* culture depends mainly on the nature of the explant and nutritive media. First of all physical state of the nutritive media presents a special significance. The negative effect of solid media no matter the type and its composition was observed. Secondly, the type of the explants presents a great importance on the basal MS (1962) and BBM (1965) media variants both solid and liquid; the thallus fragment

inoculi have not a favorable reaction which denotes that, on the one side, the explants do not confer a suitable line of contact for the nutritive exchange with the medium and, on the other side, the substances of this medium are not efficient for symbionts's nutritional requirements; therefore, the culture media without hormones do not give concludent results. The kinetin enriched media enhance symbionts's proliferation from the mycobiont and phycobiont cell suspension. In the kinetin (0.1 mg/l) enriched liquid BBM (1965), the suspension which contains both lichen symbionts has condensed in a translucent, globular mass, with a "jelly-fish" consistency; this one represents actually the hyphae network produced by the mycobiont; two months after the inoculation, this system became algae-decorated, turning in an "emerald-like" structure (Fig. 3) and, finally, a hunter green shade; three months after, both algae and the hyphae have associated in a lichen-like structure (Fig. 4). The addition of glucose and peptone has no positive effect. The tendency to crowd argues the mycobiont-phycobionts association, on the basis of affinity; it seems that the liquid culture medium contains those signals which speed the recognition and partners welding in a lichen (Ahmadjian, 1993); this aspect is illustrated by grouping the algal cells around the hyphae, as we have nicely surprised under the microscopical view of the *squash* slide (Fig. 5). Four months after, some lobe-like systems started to differentiate on the edges; also, the lichen's mass and bulk have increased and the shade was still green (Fig. 6). On liquid BBM with peptone and glucose supplements, the reaction of the grinding paste inoculi and thallus fragments was middle.

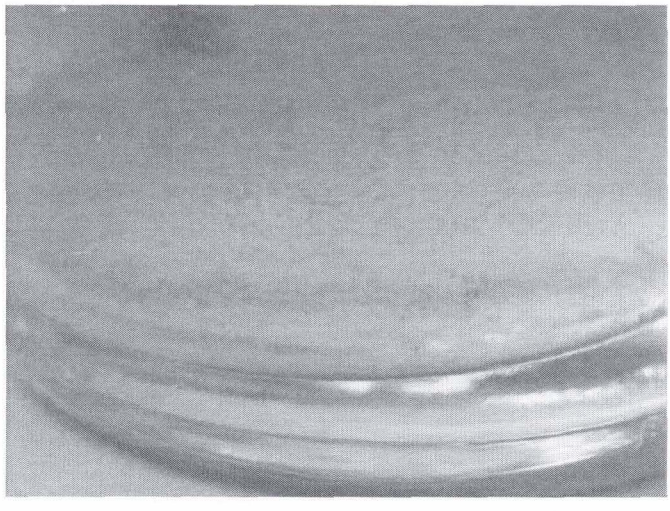

Fig. 3. Hyphae network with algae-decorated, produced by the mycobiont – two months after the inoculation time.

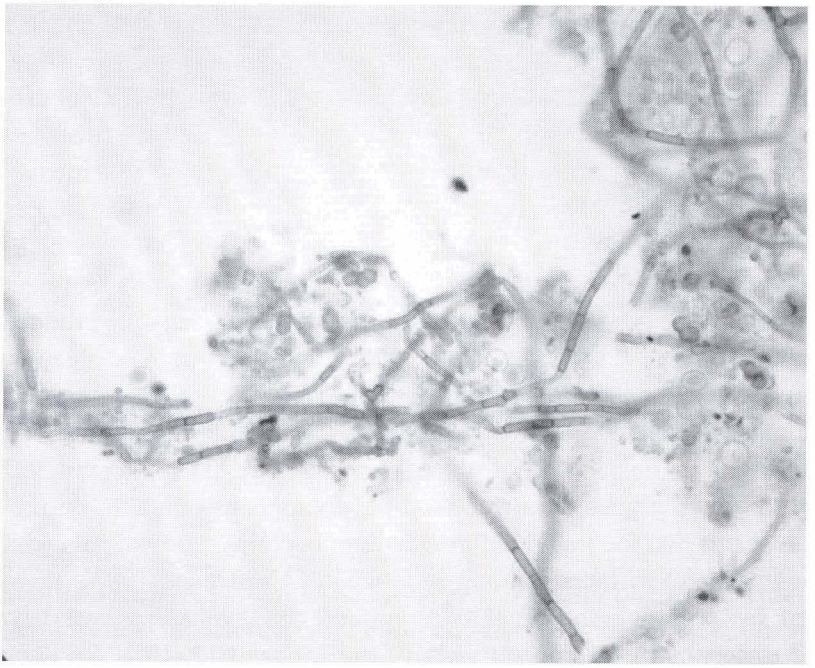

Fig. 4. Hyphae grouping around algal cells (see arrow) oc. 10, obj. 20, three months after the inoculation in BBM liquid culture medium – cotton colored slides.

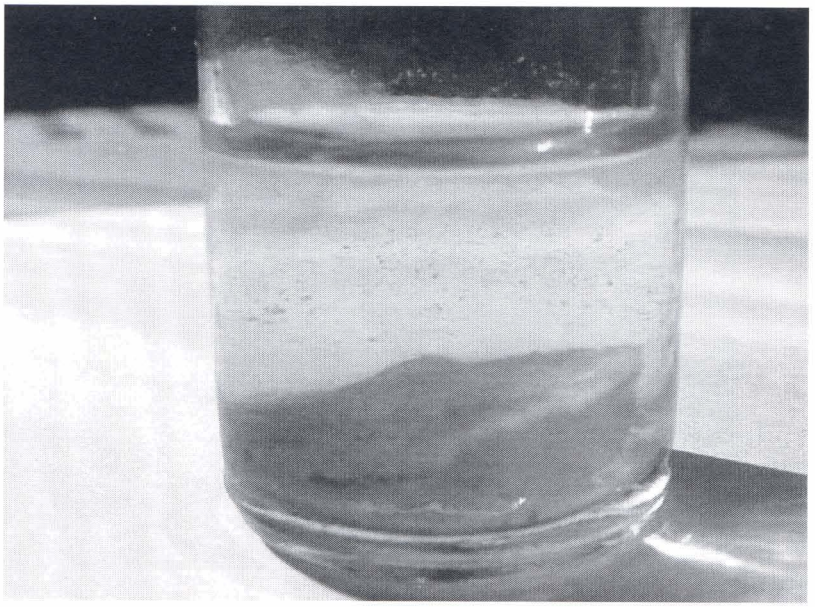

Fig. 5. Lichen-like structure with lobe-like systems on the edges.

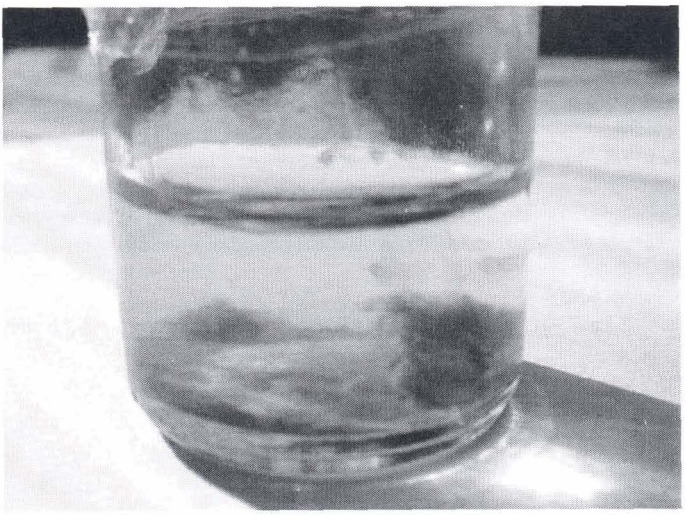

Fig. 6. Globular lichen mass.

CONCLUSIONS

These experimental results revealed that the lichen *Usnea barbata* (L.) Mott. is a responsive species to the *in vitro* conditions. The optimal culture medium was proved during these investigations to be the liquid culture medium as it is assumed to be better in the nutrients acquisition and probably related with the low oxygen concentration into the culture media. It was also observed that the type of inoculum exerts, also, an important role for *in vitro* culture success and related with this the grinding paste inoculum was considered as the most efficient for the culture conditions applied during these experiments. We may add that the cytokinins enriched media (*i.e.* kinetin 0.1 mg/l) cause a favourable reaction upon the inoculi for multiplication while addition of peptone and glucose based compounds is not benefic for the above mentioned purpose.

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