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Short Communication

Acclimatization results of micro-propagated plantlets

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This work is an analysis of the structured-functional particularities of regeneration of introduced varieties of *Vaccinium corymbosum* L. and *Vaccinium vitis-idaea* L. under *in vitro* and *ex vitro* conditions. The anatomical structures of the leaves of *V. corymbosum* and *V. vitis-idaea* cultivated under aseptical culture, greenhouse and open ground were studied. It is shown that conditioned cultivation superimposes the imprint on structure and function regeneration, structured and functional organization regeneration: a mobile system and can reform in accordance with changed condition surrounding ambiences. The differences in construction and functions of sheet plants, growing in aseptic culture, green houses or open ground are indicative of plastic sheet; an organ, capable of reconstructing its structure and function adequately under conditioned cultivation. This is theoretically a guarantor to successful adaptation of the plants under *in vitro* (the cultural container) and *ex vitro* (the greenhouse and open ground) conditions.

Key words: Aseptic culture, greenhouse, open ground, anatomical structure, blueberry, cowberry.

INTRODUCTION

In the foundation of clonal micropropagation of plants, there are two completely different stages; *in vitro* and *ex vitro*. In the first (*in vitro*), vital functions of the material being propagated occur in a closed sterile space, on the nutrient medium under strictly controlled conditions. After the regenerants are transferred from *in vitro* conditions, the second stage begins (*ex vitro* system), which is quite different from *in vitro* conditions.

In *ex vitro* conditions, the plants have to pass from heterotrophic nutrition to autotrophic nutrition conjugated with structural and functional transformation of organism under new conditions. They must adjust themselves to changeable environmental factors inherent in them.

The transition of plants from *in vitro* to *ex vitro* conditions is critical in most cases and entails death of plants. From our point of view, the comparative analysis of structural and functional peculiarities of regenerants under *ex vitro* and *in vitro* conditions will help to understand and prevent the cause of death of plants during adaptation period.

Research conducted by Brainerd et al. (1981) on leaf anatomy and water stress with plump plants under *in vitro* and *ex vitro* conditions showed that loss of water occurred three times faster in plants obtained from *in*

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vitro culture compared to those obtained from greenhouse. The thickness of palisade cells was much lower in regenerants raised under aseptical conditions than those from greenhouse and open ground.

According to researches by Grout (1975), Sutter and Langhans (1979), leaves are deprived of wax bloom in plants cultivated *in vitro* and stoma function is imperfect because of failure of open-closed mechanism. Similar conclusions about stoma functioning were obtained by Lee et al. (1988), Brainerd et al. (1982) and Wardle and Short (1983).

According to data by Bunning and Sagromsky (1948), O'Leary and Knecht (1981), Penfound (1931), stoma development is influenced by factors such as CO_2 concentration in the retort, water regime and hormone level.

The stomata of plants under *in vitro* conditions are usually open which is not true in respect with stomata *ex vitro* conditions (Brainerd and Fuchigami, 1981; Wetzstein and Sommer, 1982, 1983). In our opinion, such behaviour of stomata under *ex vitro* conditions is quite justified because in cultural retorts a very high constant relative humidity rate is kept (over 90%) and temperature and illumination degrees are not responsible to over falls because they are being controlled. Should any condition in cultural container occur, stomata reaction will follow in response to the changes of the given conditions.

From this point of view, the failure is clear overtaking some researchers seeking to interfere with efficient performance of stomata responding to conditions under which they are. For instance, the use of antitranspirants during transfer of plants from *in vitro* to *ex vitro* conditions promoted decreasing of photosynthesis caused by worsening of plant growth (Danies and Kozlowski 1974).

According to researches done by Fabbri and Sutter (1986), the leaf structure of wild strawberry formed *in vitro* culture, was characterized by a relatively thin leaf plate, under developed palisade cells, big air cavities, weakly developed cuticular integument. At the same time the leaf of wild strawberry formed under *ex vitro* conditions was differentiated into palisade and spongy tissues with a well-developed cuticular integument.

Similar results were obtained by Donnelly and Vidaver (1984) when studying raspberry leaves regenerated *in vitro*. Waldenmaier and Schmidt (1990) observed histological differences of Rhododendron leaves *in vitro* and *ex vitro* when tempering them. The differences included absence of breathing pores, weakly-structured mesophyll with leaves *in vitro*. In the *ex vitro* leaves, their anatomical structure changed: their thickness grew, the number of layers of epidermis and palisade tissue increased, and cuticle appeared. The acclimatization by low humidity rate led to a clear differentiation of the tissue into palisade and spongy mesophyll.

The objective of this study was to analyze the introduced varieties of *Vaccinium corymbosum* L. (Dixi. Bluecrop) and *Vaccinium vitis-idaea* L. (Koralle).

MATERIALS AND METHODS

The leaves of *V. corymbosum* L. (Dixi. Bluecrop) and *V. vitis-idaea* L. (Koralle) were preserved in alcohol-acetic acid (3:1). The cross sections were made in the middle part of the leaf, at microtome by histological technique and razor. The sections were cleared with chloral hydrate and then stained with Genevez and Sudan III reagents (Braune et al., 1979; Toma and Rugină, 1998; Verzar-Petri, 1979). The thickness of leaf was measured by micrometer.

The analysis of anatomical structure was realized according to previous method described by Brainerd et al. (1981), Grout (1975), Sutter et al. (1979) and Lee et al. (1988).

RESULTS AND DISCUSSION

The research on dependence of internal leaf structure under cultivating conditions showed that regenerants of *V. corymbosum (*Dixi, Bluecrop*)* and *V. vitis-idaea (*Koralle*)* cultivated under *in vitro* conditions had no clear differentiation of mesophyll into palisade and spongy tissues, had a thin leaf plate, weakly developed cuticular integument and under developed stomata apparatus entailing continuous opening of stomata and over transpiration.

The leaves developed in the greenhouse had a clear mesophyll differentiation into palisade and spongy mesophyll, had cuticular integument, and well-developed stoma apparatus enabling normal transpiration.

The leaves of plants transplanted into open ground did not differ from greenhouse leaves in general structure. They had a leaf structure clearly differentiated into palisade and spongy mesophyll, a well-developed cuticular integument and a stoma apparatus. However, it should be pointed out that the difference was observed in the change of quantitative indices of the leaf structure. Thus, leaves of plants from open ground had a thicker leaf plate, more layers of palisade tissue, longer cells, reduced volume of ductus intercellularis compared to the greenhouse and *in vitro* leaves (Table 1).

It should be pointed out that the differences in leaf structure are conjugated with their functional differences. An example is a thorough research on comparative anatomy and physiology of Asian birch (*Betula platyphylla*) cultivated in the greenhouse on aseptic culture (Smith et al., 1986). The authors concluded about the weak development of vascular system under *in vitro* conditions followed by an increased sensitivity of such plants to water stress inherent in *ex vitro* conditions.

A low intensity of photosynthesis was discovered by a very low illumination degree conjugated with the absence of clear differentiation of the leaf into palisade and spongy tissues *in vitro* culture. After the transfer of plants into *ex vitro* conditions (greenhouse) the researchers observed the increase in photosynthesis intensity and changes in leaf anatomy. In their opinion, the plants

Table 1. Quantitative indices of anatomical leaves structure of *Vaccinium corymbosum* and *Vaccinium vitis-idaea* cultivated in the aseptical culture, greenhouse and open ground*.

Grade	Aseptic culture (*in vitro*) 4000 Lx			Greenhouse >15000 Lx					Open Ground > 50000 Lx				
	Leaf thickness (µm)	The number of stomata per 1 mm²	Stoma size length x width (µm)	Leaf thickness (µm)	Palisade cofficient	Length:width of cells of palisade tissue ratio	The number of stomata per 1 mm²	Stoma size length x width (µm)	Leaf thickness (µm)	Palisade cofficient	Length:width of cells of palisade tissue ratio	The number of stomata per 1 mm²	Stoma size length x width (µm)
Vaccinium corymbosum													
Bluecrop	76±2	16±1	15x11	154±16	0.75	1.8:1	251±11	25x17	210±11	0.87	2.5:1	260±12	23x16
Dixi	85±3	16±1	15x12	173±13	0.71	1.9:1	250±9	26x16	221±12	0.9	2.7:1	265±10	24x15
Vaccinium vitis-idaea													
Koralle	91±4	19±1	16x10	286±9	0.63	2.61:1	410±20	24x15	450±19	0.86	3.31:1	430±23	21x14

*In the table no indices are shown of palisade coefficient and of palisade tissue cells with the leaves of plants from aseptic culture, since the mesophill of the leaf was not differentiated into palisade and spongy mesophills.

grown in aseptic conditions change considerably in their anatomical and physiological features compared to their double cultivated *ex vitro* conditions. The changes are accounted for by the influence of a specific environment in aseptic culture and transfer of plants into *ex vitro* conditions due to a quick recovery of metabolism resulting from normal development of plants.

According to researches by Donnely et al. (1984), Grout and Millam (1985), photosynthetical activity is lower with *in vitro* shoots compared to that of *ex vitro* shoots. In the minimum photosynthetical activity during 14 days after transfer of leaves from *in vitro* culture, it was observed that plants survived during acclimatization using the stock of metabolites. The normal recovery of structure and function occurs with the regenerants within a month after placing them under *ex vitro* conditions. To increase the survival rate of plants during adaptation it is necessary to gradually decrease the relative air humidity and increase irradiation. This increases the space occupied by palisade cells which in turn causes increase in intensity of photosynthesis.

Interesting researches were conducted by Solarova (1989) on study of round-o'clock variability of CO_2 concentration in cultivating retorts, where regenerants plants cultivated were obtained from leaf pieces. It turned out that CO_2 concentration in retorts increased in dark period and was connected to the regenerant size and sucrose content in the medium. The concentration in retorts decreased in light period and the illumination reached the compensation point in 3-4 h despite the low illumination degree (100 µmol.m^{-2}.s^{-1}). The author made a conclusion that the low CO_2 concentration in closed retorts for cultivation of regenerant plants induces different growth.

Therefore, the decreased CO_2 concentration is one of the low photosynthetical intensity observed with regenerants plants *in vitro* culture. The CO_2 concentration is increased by transfer of plants to *ex vitro* conditions, causing an increase of intensity of photosynthesis followed by growth acceleration.

On the foundation of comparative analysis of structural and functional features of the regenerants under *in vitro* and *ex vitro* conditions based on written sources and results of our own researches, we came to a conclusion: 1) that *in vitro* and *ex vitro* cultivating conditions leave imprint on structure and functions of regenerants; 2) structural and functional organization of regenerants is a mobile system able to be transformed in accordance with the changed environmental conditions. That means that the differences in structure and function of plant leave growth under aseptic culture, greenhouse or open ground testify to the flexibility of the leaf – the organ able to transform its structure and function according to the cultivating conditions. This is theoretically the guarantor of a successful adaptation of plants when transferring them from *in vitro* to *ex vitro* conditions.

In practice, we managed to avoid losses of plant material at the critical point due to using techniques based on conclusions confirmed by the results of experimental researches. This was proved by our observations over adaptation process of introduced species of *V. corymbosum* (Dixi, Bluecrop, Herbert, Rancocas, Covill, Early blue*)* and *V. vitis-idaea (*Koralle, Masovia,

Erntedank, Erntecrone, Erntezegen*)* when transferring them from *in vitro* to *ex vitro* conditions.

To prevent death of material from over transpiration (refers not only to *V. corymbosum* and *V. vitis-idaea*) caused by the reasons known to us: 1) the humidity drop *ex vitro* conditions, 2) imperfect structural and functional organization of the leaf in terms of *ex vitro* conditions, there is need firstly to increase the turgor of regenerants to its maximum value. It is achieved by plunging of the material into retort containing distillated water for 5-6 h.

The second essential condition is to keep high humidity rate in the greenhouse (not under 90%) and removal of strong air flows that is elimination of any wind since it dries up leaves because of quick evaporation. Absence of wind and high humidity rate will cause steam pressure gradient between leaves and air. It is essential to create *in vitro* identical conditions in the greenhouse in the first two to three weeks of regenerant cultivation (before root for-mation). It means to strictly control humidity rate, keep temperature similar to that when cultivating plants *in vitro* conditions and relatively low illumination degree (500 lx). Thus, the high air humidity will not cause intensive transpiration preventing the plant from fading. High temperature (25°C) and low illumination degree (500 lx) favour low intensity of photosynthesis and stop regenerant growth. The stock of metabolites with the regenerant will be utilized for root formation. After root formation, it is necessary to gradually decrease the air humidity around the regenerant and increase the illumination degree. This will complete the structural transformation of the leaf: the cuticular layer will appear, the cells of epidermis will change their shape, the mesophyll of the leaf will change its texture. The leaves will acquire features of xeromorphic structure and the plant will not be frightened by the low air humidity and even by strong wind characteristic under open ground conditions.

The procedures mentioned strictly and implemented by us when transferring the introduced species of *V. corymsosum* and *V. vitis-idaea* from *in vitro* to *ex vitro* conditions allowed us to preserve the viability of the plants and to secure their 100% survival and adaptation.

To sum it up, it can be concluded that the successful adaptation of regenerant plants when transferring them from *in vitro* to *ex vitro* conditions depends, on one hand, on our theoretical knowledge, results of experimental researches and, on the other hand, on the strict observance of simple techniques. The confirmation is a case of 100% adaptation of regenerant plants of introduced species of *V. corymbosum* and *V. vitis-idaea* not only under greenhouse conditions but also under open ground conditions.

Conflict of interests

The authors have not declared any conflict of interest.

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