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PHYSIOLOGICAL CHARACTERISTICS OF *IN VITRO* AND FIELD CULTIVATED *LEUCOJUM AESTIVUM* L. PLANTS

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Summary. A living collection of *Leucojum aestivum* L. (Amaryllidaceae) was established at the Institute of Botany, Sofia, in 2001. *In vitro* cultures originating from a single mother bulb have been maintained since 2003 on MS-based agar-solidified medium supplemented with 30 g L^{-1} sucrose, 2 mg L^{-1} BAP and 0.15 mg L^{-1} NAA under equal temperature and light conditions in plastic containers. Part of the regenerated plantlets were successfully adapted *ex vitro* and planted in open-air trenches in 2007. Uniform shoot clumps were selected and grown for seven weeks during the vegetation peirod in spring 2009 on solid and in a liquid medium with the same compostion. Main physiological characteristics of the field cultivated plants and plantlets from long-term *in vitro* cultures were compared. Leaves were taken from field grown and field adapted plants, and in vitro obtained plantlets on solid and in liquid media, all genetically identical. The photosynthetic rate of field grown plants was twice higher than that of in vitro plantlets. Moreover, the results for the plants adapted to open-air conditions were similar to those initially planted on the field, whereas the photosynthesis and the biomass accumulation were more expressed by the in vitro plantlets cultured in a liquid medium compared to those grown on agar-solidified one. The liquid shoot-clump culture of *L. aestivum* was determined as a suitable system for *in vitro* alkaloid production.

***Key words*:** *in vitro* culture, photosynthetic activity, summer snowflake.

***Abbreviations*:** BAP – 6-benzylaminopurine; Chl – chlorophyll; NAA – α-naphthalene-acetic acid; PSII – photosystem II.

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INTRODUCTION

A living collection of *Leucojum aestivum* L. (Amaryllidaceae) representative for the species in Bulgaria was established in 2001 at the Institute of Botany in Sofia, within the framework of project SfP 974453-Bioproduction, aiming at conservation of the genetic biodiversity and selection of high galanthamine productive individuals. Plant propagation enhancement by *in vitro* techniques and alkaloid biosynthesis in cultures were previously reported (Bogdanova et al., 2009; Stanilova et al., 2009). Genotype was proved to be most important for successful alkaloid production. In order to reveal the impact of the cultivation conditions on plant metabolism, we compared the main physiological characteristics of field cultivated plants and plantlets from long-term *in vitro* cultures, using only genetically identical plant individuals.

MATERIALS AND METHODS

Plant material

Green leaves of *L. aestivum* were used for analyses during the spring vegetation in 2009. All samples were taken from genetically identical plants obtained by *in vivo* or *in vitro* vegetative propagation of a single plant cluster of galanthamine type originated from the population Arkutino. Field growing plants have been cultivated in the living collection for eight years. *In vitro* culture was initiated in 2003 from a single mother bulb belonging to the cluster and multiplied by clonal micropropagation (clone La-5.2) as previously discribed (Bogdanova et al., 2009). Cultures have been maintained for six years on MS-based medium (Murashige and Skood, 1962) solidified with 6 g L^{-1} Plant agar, supplemented with 30 g L^{-1} sucrose, 2 mg L^{-1} BAP and 0.15 mg L^{-1} NAA, under equal conditions: 23±2°C, a 16/8 h light/dark period and light intensity of 40.5 μmol m^{-2} s^{-1}, in Vitro Vent plastic containers (Duchefa, NL). A part of the regenerated plantlets were adapted *ex vitro* and planted in open-air trenches in 2007. Uniform shoot clumps were grown for seven weeks in spring 2009 on solid and in a liquid medium with the same composition. At the end of the period leaves were taken for analyses from all four types of plants: field growing, field adapted, and *in vitro* grown on solid and in liquid media.

Chlorophyll fluorescence

Chlorophyll fluorescence emission from the upper leaf surface was measured with a pulse amplitude modulation fluorometer (PAM 101-103, Walz, Effeltrich, Germany). The initial fluorescence yield in weak modulated light (0.075 μmol m^{-2} s^{-1} PPFD), F_0, and maximum total fluorescence yield emitted during a saturating white light pulse (1 s, over 3500 μmol m^{-2} s^{-1} PPFD, by Schott KL 1500 light source), F_m, were determined. The leaf disc (10 mm diameter) was then illuminated with continuous red light (100 μmol m^{-2} s^{-1} PPFD). The short pulses (with 20 s interval) on the background of a red light were used to obtain the fluorescence intensity F_m' with all PSII reaction centers closed in any light-adapted state. Induction kinetics were registered and analyzed with the program FIP 4.3, written by Tyystjarvi and Karunen (1990).

Oxygen evolution

Oxygen evolution rate was determined using a leaf disc electrode (Type LD2/2, Hansatech, U.K.). It was measured at 800 μmol m^{-2} s^{-1} PPFD at saturating CO_2 concentration (provided by a carbonate/ bicarbonate buffer).

Scanning electron microscopy

Leaves of field growing plants and submerged leaves of liquid cultures were prefixed in 5% buffered glutaraldehyde (0.1 M phosphate buffer, pH 7.2) for 2 h at room temperature. After dehydration through a graded ethanol series, samples were dried with a CO_2 critical-point drying system sputtered with gold and observed with a scanning electron microscope (JSM 35).

RESULTS

The maximum quantum efficiency of PSII estimated by the variable to maximum Chl fluorescence ratio (F_v/F_m), in plants growing in the field and in those adapted to *in vivo* conditions was very similar (Fig. 1). The F_v/F_m ratio was slightly lower in plantlets growing *in vitro* in liquid medium, whereas it declined by more than 10% in those grown on agar. The quantum efficiency of the electron transport through PSII (ΦPSII) in plantlets grown in liquid media and on agar was reduced by 10% and 35%, respectively, as compared to those grown in the field. ΦPSII is related to the photochemical fluorescence quenching, qP and the relative photochemical efficiency of open PSII reaction centers (F_v'/F_m') (Genty et al., 1989). The decline in ΦPSII observed in plantlets on agar was due to a reduction of qP (by 10%) and especially of F_v'/F_m' (by about 30%). The lower photochemical activity of PSII in plantlets grown on agar was not due to lower chlorophyll content. Actually, their chlorophyll content was higher compared to the plantlets grown in the liquid medium, but the differences were not statistically significant (data not shown).

From the slow component of the Chl fluorescence induction kinetics of pre-darkened leaves, the ratio of Chl fluorescence decrease to the steady state (F_s) Chl fluorescence R_{Fd} [$R_{Fd} = (F_{max} - F_s)/F_s$] can be determined. The Chl fluorescence decrease ratio covers the whole process of photosynthesis, including the full induction period, the transition of the photosynthetic apparatus from the non-functional state 1 to its functional state 2, and the photosynthetic CO_2 fixation (Lichtenthaler and Miehé, 1997). R_{Fd} values permit fast screening of the photosynthetic activity and vitality of plants. Our results showed that compared to plants grown in the field, R_{Fd} values measured in plantlets grown on agar and in liquid media was lower by 57% and 20%, respectively (Fig. 1). The decreased photochemical activity in plantlets grown on agar correlated with an increased proportion of light not used for photochemistry (LNU) (Fig. 1).

The rate of photosynthetic oxygen evolution was lower in plantlets grown *in vitro* than *in vivo* (Fig. 2). It was lower in plantlets grown on agar and liquid medium by about 70% and 50%, respectively.

The dry matter of the submerged leaves was lower compared to the leaves of the plants grown at open air: 8.3 and 12.7 DW/FW%, respectively; however, no hyperhydricity was detected in the

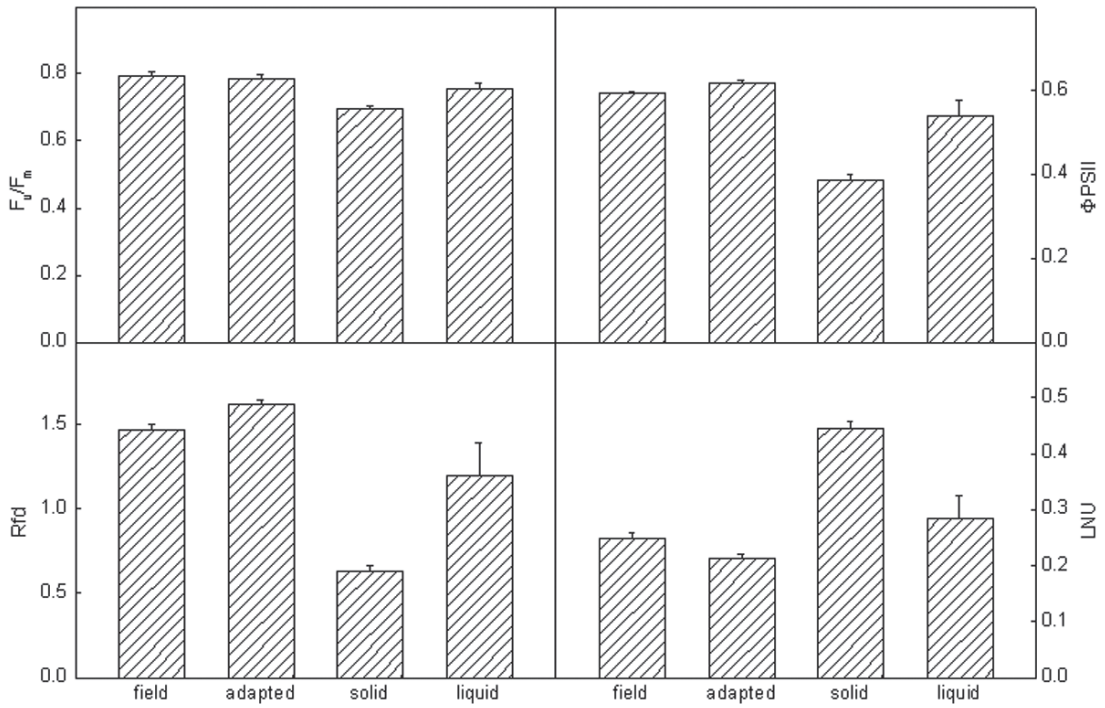

Fig. 1. Effects of the growth conditions on the maximum quantum efficiency of PSII (F_v/F_m), the actual quantum yield of PSII electron transport in the light-adapted state (ΦPSII), the ratio of Chl fluorescence decrease to the steady state Chl fluorescence R_{Fd} [$R_{Fd} = (F_{max} – F_s)/F_s$] as a measure of photosynthetic activity and vitality of plants, and the light not used for photochemistry (LNU) in *Leucojum aestivum*.

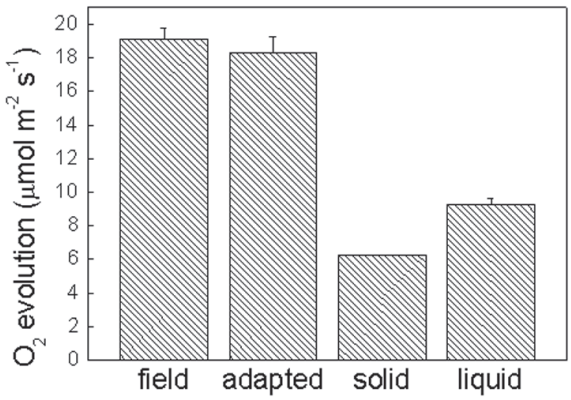

Fig. 2. Oxygen evolution rate in *L. aestivum*, cultivated in different conditions.

liquid cultures. The state of the stomata apparatus differed depending on the cultivation conditions (Fig. 3). The leaves of the field grown plants had stomata apparatus with normal structure and function, whereas those of the submerged leaves from the liquid *in vitro* culture showed some structural modifications resulting in permanent closure and abnormal functioning.

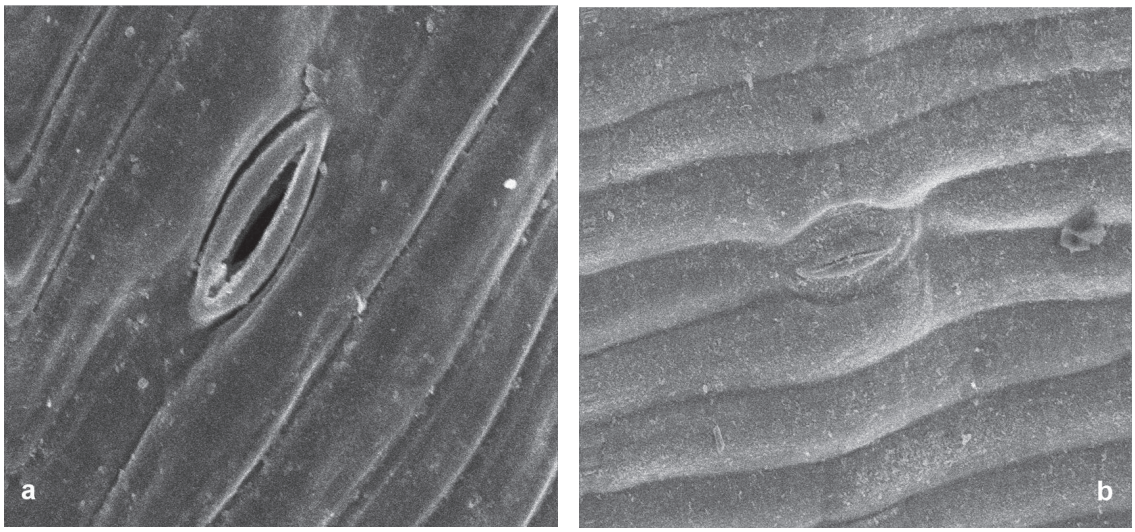

Fig. 3. Stomata in *L. aestivum* leaves in surface views: (a) leaf of field growing plant; (b) submerged leaf of in vitro liquid culture.

DISCUSSION

The photosynthetic effectiveness and the plant vitality depended on the cultivation conditions. Although the photosynthetic rate of the *in vitro* plantlets was twice lower compared to that of the field grown plants, it was recovered in the *ex vitro* adapted plants. Since the chlorophyll content of the plantlets from the liquid culture was very similar to that of the field growing plants, the reasons for the decrease of the photosynthetic activity *in vitro* was rather related to the abnormal state of the stomata. In general, the regulation of the water vapour and carbon dioxide exchange *in vitro* is difficult because of the high relative humidity in the culture vessels. The closure of the stomata in the liquid medium indicated cessation of their functioning. Usually, *in vitro* plants are not fully autotrophic and need addition of carbon-containing compounds in the medium. Our preliminary experiments showed that the biomass accumulation was about twice faster in the liquid cultures compared to that of the plantlets grown on agar-solidified medium, which could be due to the large surface contact of the submerged leaves with the nutrients of the medium. In spite of the lower dry matter of the submerged leaves, they were massive and vigorous, which probably could explain their higher photochemical activity mesured per leaf discs of equal surface of 10 mm diameter. On the other hand, the normal state of stomata and the high chlorophyll contents of the field growing and the *ex vitro* adapted plants ensured their regular photosynthesis.

These results confirmed the high quality of the *ex vitro* adapted plants of *L. aestivum,* thus defining *in vitro* micropropagation as a very appropriate step for propagation enhancement of the selected high galanthamine productive genotypes. In addition, shoot-clump *L. aestivum* liquid cultures were determined as a suitable system for rapid biomass accumulation, which is essential for *in vitro* alkaloid production.

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