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**Authors' contributions**

AR and CF performed tissue culture work and measured antioxidant activities, helped in experiment design; FK performed tissue culture and the measurement of antioxidant activities, mainly for *S. lutea*; SG made essential work in data analysis; LP helped in explant sampling; MHM and KA made essential contributions in native gel electrophoresis for the assay of peroxidase activities; CM designed experiments and wrote the manuscript; AR and CF contributed equally to the present work

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**Competing interests**

No competing interests have been declared.

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## ORIGINAL RESEARCH PAPER

# Production and antioxidant capacity of tissue cultures from four Amaryllidaceae species

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**Abstract**

The aim of this study was (*i*) to produce tissue cultures capable of efficient plant regeneration from European naturally occurring protected and/or pharmacologically important Amaryllidaceae species and (*ii*) to test them for antioxidant activities in order to select tissue cultures that scavenge efficiently oxygen radicals. Bulb explants were collected from *Galanthus woronowii*, two *Leucojum* species, and *Sternbergia lutea*. *Leucojum* species were Hungarian isolates. Mostly  $\alpha$ -naphthalene acetic acid (NAA) and benzyladenine (BA) were used as growth regulator combinations for the induction and maintenance of tissue cultures and further antioxidant activity studies. *Galanthus woronowii* and *L. vernum* cultures produced shoots or whole plants via micropropagation (callus stage was observed only sporadically and callus tissue did not contribute to regeneration), whereas *L. aestivum* and *S. lutea* produced efficiently whole plants or multiple shoots via embryogenic calli. Total phenolic content, % inhibition of ABTS radical (ABTS<sup>+</sup>) cation, and peroxidase activities on native polyacrylamide gels were studied and showed differences between cultures. No relationship could be detected between polyphenol content / radical scavenging capacities and H<sub>2</sub>O<sub>2</sub> reducing enzyme activities. For *G. woronowii*, *S. lutea*, and a culture line of *L. vernum*, polyphenol content and ABTS<sup>+</sup> cation scavenging activities were high and for *G. woronowii*, comparable to organs of the native plants used as explant sources. Bulbs of native plants showed low radical scavenging activities in general. For *L. vernum* and *L. aestivum* tissue cultures grown in the presence of NAA as the sole growth regulator, ABTS<sup>+</sup> cation scavenging showed low values. Enzymatic antioxidant (pyrogallol peroxidase) activities were low for all cultures and organs of native plants. This study shows the species conservation value of these cultures and highlights the high antioxidant capacity of *G. woronowii* and *S. lutea*, attributed to the presence of non-enzymatic scavengers.

**Keywords**

Amaryllidaceae; micropropagation; somatic embryogenesis; free radical scavenging; pyrogallol peroxidase

**Introduction**

Many wild-growing Amaryllidaceae species are protected or even endangered in Hungary / Central Europe in particular and Europe in general, being included in the red list of threatened species (Convention on International Trade in Endangered Species of Wild Fauna and Flora – CITES), due to deterioration of their habitats and

extensive collection [1,2]. Thus, their conservation is of major importance. Moreover, antioxidant capacity and its physiological background is poorly studied in Amaryllidaceae tissue cultures, even though many species of this family are known for their medicinal value. The most important metabolites studied in this respect are the Amaryllidaceae alkaloids like galanthamine and lycorine, but the activity of types of other low-molecular weight bioactive compounds in cell-free extracts (like total polyphenolics) are much less known [3,4]. For plants growing in their native habitat, antioxidants and free radical scavenging capacities of Amaryllidaceae were studied and high values indicating possible pharmacological uses were occasionally reported. Although *Curculigo orchioides*, an amaryllid, was not involved in this study, it is worth mentioning that its methanolic extracts have significant antioxidant and protective effects in diabetic rats [5]. Curculioside, a phenolic compound of this plant, has a significant antioxidative capacity and as such it was suggested to be potentially used in the treatment of osteoporosis [6].

Tissue culture methods have been employed for a relatively large number of amaryllids. These in vitro cultures were used for mass plant propagation for species conservation / germplasm preservation and alkaloid production purposes. Particularly, there is no need to harvest plants from their natural habitats for the production of biologically active compounds, because tissue culture procedures could supply raw material for such purposes. Some important examples are *Galanthus elwesii*, *Leucojum aestivum*, and *Narcissus pseudonarcissus* [7,8].

The present study involves tissue cultures of *Galanthus woronowii*, *Leucojum vernum*, *L. aestivum*, and *Sternbergia lutea* (Amaryllidaceae) individual genotypes. Explants of *Leucojum* species were of Hungarian and *S. lutea* of Albanian origin (see “Material and methods”). Explants of *G. woronowii*, a species native in the Caucasian Mts, were from commercial source. In the light of the presented state-of-the art, the main objectives of the present study were:

- to establish and maintain tissue cultures capable of mass shoot / whole plant regeneration of the above-mentioned Amaryllidaceae genotypes that are mostly both endangered and potential producers of pharmaceuticals. *Leucojum aestivum* was widely studied for tissue culture induction. Concerning *L. vernum*, there is a report on somatic embryogenesis [9]. However, none of these *Leucojum* tissue cultures were studied for their antioxidant capacities. For *G. woronowii* and *S. lutea*, we have found no comprehensive description of tissue cultures in previous literature.
- to analyze for the first time phenolic content, free radical scavenging, and enzymatic antioxidant activities of the above tissue cultures in order to select culture lines bearing high antioxidant capacities, with potential pharmacological importance.

## Material and methods

### Initiation and maintenance of tissue cultures

The plant material used for tissue cultures were bulb explants (containing bulb scales and proximal portions of aerial shoots) of *G. woronowii* Losinsk. (commercial source), *Leucojum vernum* L. (Magosliget, NE Hungary / Botanical Garden, University of Debrecen) collected in 2011, *L. aestivum* L. (Márokpapi, NE Hungary, collected in 2014), and *Sternbergia lutea* (L.) Ker-Gawl. Ex Spreng (Shalës, Albania) collected in 2013. Explants were surface sterilized as described [10] and transferred to MS basal medium supplemented with 2% (w/v) sucrose, Gamborg’s vitamins [11,12] and different growth regulator combinations involving  $\alpha$ -naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), and indole-3-acetic acid (IAA) as auxins and benzyladenine (BA) as a cytokinin. In general, 2–4 explants were transferred to Petri dishes containing culture media solidified with 0.8% (w/v) Bacto-agar (Difco, USA). Physical (light and temperature) conditions were as described [10]: 14/10 (light/dark) photoperiod (white fluorescent light,  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and  $22/18 \pm 2^\circ\text{C}$ . Regeneration potential of tissue cultures was evaluated after 32–38 days of subculture in at least five independent experiments, followed by their analysis for antioxidant capacities (see sections below).

## Determination of total phenolics content and antioxidant capacities

For the analysis of polyphenol content, free radical scavenging capacities, and peroxidase activities, the nature of samples depended on the type of regenerants obtained: whole plants for *L. vernum* and *L. aestivum*, and shoots for *G. woronowii* and *S. lutea*, where root formation was sporadic or did not occur at all (shoots were 2–3 cm in length, while root length was 2–4 cm, where appropriate). In addition, *S. lutea* embryogenic calli were analyzed. For the determination of phenolic contents and free radical scavenging capacities, samples of tissue cultures were lyophilized in a Christ Alpha 1-2 LD freeze dryer (Martin Christ, Germany) and extracts were prepared in 30% (v/v) methanol. Samples were incubated in a water bath for 15 min at 70°C for improving the efficiency of extraction, then subjected to prolonged tissue disrupting with a Disruptor Genie (Bohemia, USA) and centrifuged (15 000 rpm; Heraeus Biofuge), then supernatants were used. This method was efficient as proven by the absence of polyphenols after a second extraction from the same lyophilisate. For the photometric procedures presented below (assay of total phenolic content and ABTS\* inhibition), the ratio between samples and 30% methanol was set up to obtain a supernatant containing 25 mg DW mL<sup>-1</sup> plant material. These supernatants were used for spectrophotometric assays, where each mL of reaction mixtures contained 0.5 mg DW of plant material for achieving the most accurate results.

Total phenolic content was assayed by a modified Folin–Ciocalteu (FC) method as described [13,14]. Briefly, 8-fold diluted FC reagent and a final concentration of 233 mM Na<sub>2</sub>CO<sub>3</sub> was used in the assay. A<sub>720</sub> of samples was measured with a Shimadzu UV-VIS 1602 spectrophotometer (Shimadzu, Japan) and this photometer was used for the assay of free radical scavenging capacities as well. Gallic acid was used as standard and total phenolic content of plant samples was expressed as gallic acid equivalent (GAE), mg g<sup>-1</sup> DW.

Free radical scavenging capacity was measured by the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS\* cation) inhibition assay as described [15,16]. ABTS was purchased from Sigma-Aldrich, USA. Briefly: for the ABTS\* cation inhibition, ABTS radical was produced with potassium persulfate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>) and the solution diluted with ethanol to reach an A<sub>734</sub> value of 0.7. Plant samples were added thereafter. The percentages of radical inhibition were calculated according to the following formula:  $[(A_0 - A_{\infty})/A_0] \times 100$ , where A<sub>0</sub> is the absorbance of ABTS\* cation at the start of the scavenging reaction and A<sub>∞</sub> is the absorbance at the 6th minute of the reaction. Ascorbic acid (AA) and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were used as standards. AA equivalents and Trolox equivalent antioxidant capacities (TEAC) were calculated, based on the extrapolation of ABTS\* cation inhibition values to the calibration curves of standards.

Enzymatic H<sub>2</sub>O<sub>2</sub> scavenging (peroxidase) activities were analyzed on native 7.5% polyacrylamide gels. Crude enzyme extracts of plant tissue samples were prepared in a buffer containing 150 mM NaCl (Reanal, Hungary), 14.6 mM 2-mercaptoethanol (2-ME, Sigma-Aldrich) in 10 mM Tris-HCl (Sigma-Aldrich) pH 8.0 [17]. Buffers were added at a 2:1 buffer: tissue FW ratio. After repeated centrifugations at 15 000 rpm with a Heraeus Biofuge, the protein contents of 10 µL of supernatants were checked with the Bradford method [18], followed by their loading onto the gels. Each sample contained 3.5–4 mg (FW equivalent) of plant material. Guaiacol peroxidase activity gels were prepared and analyzed as described previously [19]. For the detection of pyrogallol peroxidase bands, native gels were incubated in 50 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.5, then stained for 15–30 min in the same buffer containing 20 mM pyrogallol and 5 mM H<sub>2</sub>O<sub>2</sub>. The oxidation product (purpurogallin) was detected as a pattern of dark-brown colored bands. Root extracts of horseradish (*Armoracia rusticana*) known for high peroxidase activities [20] were used as positive controls. Their activity was considered as 100% and all activities of Amaryllidaceae tissue cultures were compared to this value.

Gels were analyzed with the aid of CP Atlas software. Total band intensities were calculated on the basis of peak areas given by the enzyme activities, then plotted with the aid of Systat Sigma Plot 10.0 software.

Peroxidase activities were also assayed by a spectrophotometric method using 3 µL of the same enzyme extracts as for native activity gel electrophoresis, by a method

described previously [21]. Since 2-ME is known to inhibit the activity of certain peroxidases as revealed by photometric studies [22], the effects of the presence and absence of 2-ME in the extraction buffer on enzyme activities were studied.

For free radical scavenging capacities and peroxidase activities, at least five independent experiments have been performed, and for the former, two parallel measurements were made for each plant sample per experiment.

## Results

### Establishment and maintenance of tissue cultures

Culture media for efficient callus production and/or shoot/plant regeneration were designed as based on our previous work [10] for the establishment of *Galanthus nivalis* tissue cultures. Between 30 and 45 days of culture were necessary for the initiation of callus or shoot production. Tissue culture types and the efficiency of plant/shoot/bulblet regeneration are presented on Tab. 1.

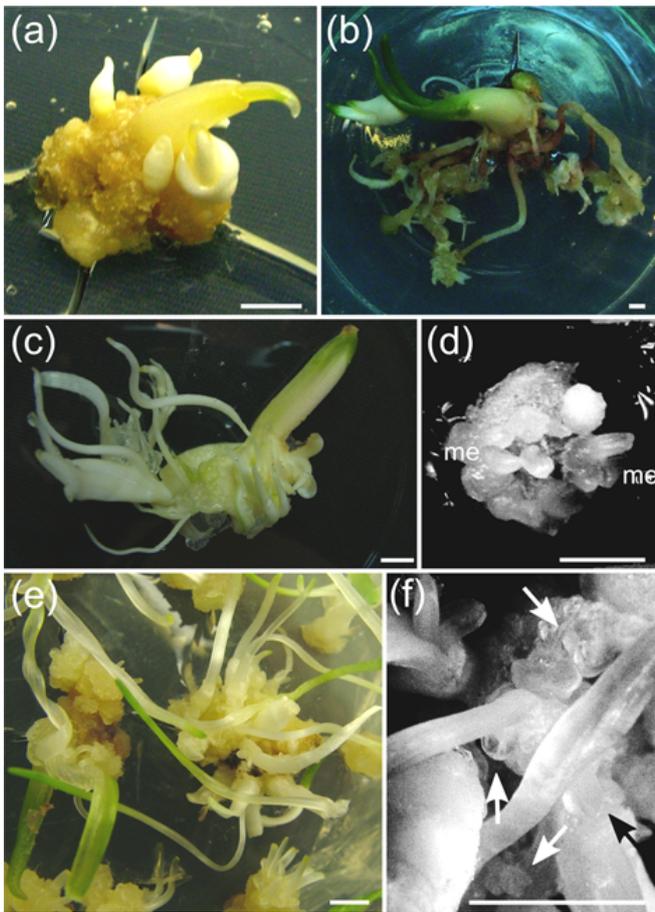
For *G. woronowii*, bulb explants produced aerial shoots and bulblets directly via bulb morphogenesis and somatic embryo formation was not observed in the presence of 2 or 10 mg L<sup>-1</sup> NAA in combination with 1 mg L<sup>-1</sup> BA. Non-embryogenic calli and rooting could be observed sporadically (Fig. 1a). A combination of 2 mg L<sup>-1</sup> NAA with 2 mg L<sup>-1</sup> IAA induced intensive shooting with no callus or root formation, while 2 mg L<sup>-1</sup> NAA used together with 2 mg L<sup>-1</sup> IBA induced intensive rooting together with shoot differentiation (data not shown). The combination of 2 mg L<sup>-1</sup> NAA with 1 mg L<sup>-1</sup> BA lead to 100% efficiency of bulblet formation (Tab. 1).

For *L. vernalis*, bulb explants produced whole plants with complete shoot and root system and bulblets directly (i.e., without an intermediary callus stage) on 1–2 mg L<sup>-1</sup> NAA or a combination of 2 mg L<sup>-1</sup> NAA with 1 mg L<sup>-1</sup> BA, and non-embryogenic calli formed sporadically (Fig. 1b). Rooting was promoted as well, when 2 mg L<sup>-1</sup> NAA was combined with 2 mg L<sup>-1</sup> IBA (data not shown). Somatic embryos were not detected. Bulblet regeneration frequency was high (Tab. 1). In contrast, bulbs of *L. aestivum* developed into embryogenic calli on 2 and 10 mg L<sup>-1</sup> NAA. After 14 days of subculture of established calli, efficient production of mature somatic embryos was achieved (Fig. 1d). Further culture of embryogenic calli led to mass production of shoots and on 2 mg L<sup>-1</sup> NAA, rooting of somatic embryo derived shoots was promoted (Fig. 1c).

**Tab. 1** Regeneration potential of cultures used for subsequent studies of antioxidant capacities. Mean  $\pm$ SE values for 32–38-day-old cultures are presented;  $n \geq 10$  per experiment, at least five independent experiments were performed.

Culture line, PGR content	Type of culture	Number of regenerants <sup>a</sup>	Bulblet formation frequency <sup>b</sup>
1a. <i>Galanthus woronowii</i> , 2 mg L <sup>-1</sup> NAA + 1 mg L <sup>-1</sup> BA	Sporadic callus, shoot production via micropropagation	2.33 $\pm$ 0.84	100.00
2a. <i>Leucojum vernalis</i> , 2 mg L <sup>-1</sup> NAA	Sporadic callus, whole plant production via micropropagation	1.25 $\pm$ 0.25	37.50 $\pm$ 12.5
2b. <i>L. vernalis</i> , 2 mg L <sup>-1</sup> NAA + 1 mg L <sup>-1</sup> BA	No callus, whole plant production via micropropagation	1.62 $\pm$ 0.18	37.50 $\pm$ 12,5
3a. <i>L. aestivum</i> , 2 mg L <sup>-1</sup> NAA	Embryogenic callus, whole plant regeneration	2.85 $\pm$ 0.39	7.69 $\pm$ 3.01
4a, b. <i>Sternbergia lutea</i> , 2 mg L <sup>-1</sup> NAA + 1 mg L <sup>-1</sup> BA	Embryogenic callus, shoot regeneration; whole plant regeneration after rooting of regenerated shoots on PGR-free culture medium	3.53 $\pm$ 0.67	28.44 $\pm$ 8.1

<sup>a</sup> Per callus where embryogenic calli are produced (3a, 4a, b); per micropropagation units, where callus was sporadically formed or did not develop at all (1a, 2a, b). Micropropagation unit is a tissue culture cutting containing one macroscopically visible shoot primordium. <sup>b</sup> Is the percentage of shoots / whole plants that produce bulblets.



**Fig. 1** Characteristic cultures involved in this study. Cultures shown on **a, b, e, f** were grown on a culture medium with 2 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BA, while cultures shown on **c, d** were grown in the presence of 2 mg L<sup>-1</sup> NAA. **a** Typical shoot culture of *Galanthus woronowii*. **b** Micropropagated *Leucojum vernum* plants. **c** *Leucojum aestivum* shoots originated from embryogenic calli. **d** Development of *L. aestivum* somatic embryos on a callus. me – mature somatic embryo. **e** *Sternbergia lutea* calli with regenerated shoots. **f** Detail of a *S. lutea* embryogenic callus with somatic embryos (arrows). These calli were capable of efficient shoot regeneration. Scale bars: 2.5 mm.

Bulblet regeneration frequency was relatively low (Tab. 1). Between 1 and 2 mg L<sup>-1</sup> NAA together with 1 mg L<sup>-1</sup> BA induced the formation of embryogenic calli with shoots, but no rooting could be observed (data not shown).

For *Sternbergia lutea*, bulb-derived embryogenic calli with somatic embryos developed on media containing 2 and 5 mg L<sup>-1</sup> NAA with 1 mg L<sup>-1</sup> BA. Somatic embryos converted to shoots (Fig. 1e,f) with high bulblet regeneration frequency (Tab. 1). Rooting occurred, when these shoot cultures were transferred to growth regulator free medium (data not shown).

To summarize tissue culture results, a common culture medium suitable for shoot or whole plant production contained 2 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BA for *G. woronowii*, *L. vernum*, and *S. lutea*. Two mg L<sup>-1</sup> NAA was used for this purpose in *L. aestivum* and one culture line of *L. vernum*. The frequency of regenerants was between 1.25 ± 0.25 and 3.53 ± 0.67 per callus or micropropagation unit (a tissue culture cutting containing one macroscopically visible shoot primordium) (Tab. 1) indicating high regeneration potential. Between 32- and 38-day-old cultures grown on these media were used for the study of antioxidant capacities.

#### Total phenolic contents and antioxidant capacities

Total phenolic content is considered relatively high, when GAE values are in the range of 12–20 mg g<sup>-1</sup> DW or higher [15,23]. According to this, total phenolic content was high for *Galanthus woronowii* shoots obtained in vitro, *Leucojum vernum* grown on 2 mg L<sup>-1</sup> + 1 mg L<sup>-1</sup> BA, *L. aestivum* embryogenic callus derived shoots and cultures of *Sternbergia lutea* (Tab. 2). Low phenolic content was detected in *L. vernum* plantlets in vitro grown on 2 mg L<sup>-1</sup> NAA as the sole growth regulator. For *G. woronowii* and *L. aestivum*, data similar to their corresponding tissue

cultures were obtained for shoots of native plants (bulbs of these plants were used for tissue culture production). In case of *L. vernum* and *S. lutea*, native leaves were characterized by higher polyphenol content, than their corresponding tissue cultures (obtained from bulbs of these plants). However, tissue cultures stated above still showed high polyphenol contents). Native bulbs showed lower phenolic contents, than shoots in general (Tab. 2).

For ABTS\* scavenging, 1 mg DW of plant material is considered as having high antioxidant capacity, when inhibition values (decrease of radical levels) are ≥60%. The method itself is a good indicator of radical scavenging capacity of a given sample as compared to various other methods: DPPH\*, FRAP, ORAC, etc. [15,16,24,25]. According to this, *G. woronowii* and *S. lutea* cultures were characterized by high inhibition values (>70 %) and 0.5 mg DW of tissue culture samples extracted in 30% methanol had higher antioxidant capacities than 2 mg (11.57 μM) ascorbic acid in the assay (Tab. 2). Even though values of antioxidant capacities depend on assay conditions, when we compare our results to literature data [25], TEAC values for ABTS\* scavenging show high antioxidant capacities for these two cultures (Tab. 2). Thus, their high antioxidant capacity was accompanied by high phenolic contents, moreover these values were comparable to leaves of native plants (Tab. 2). Micropropagated plants of *L. vernum* grown on a culture medium containing 2 mg L<sup>-1</sup> NAA and

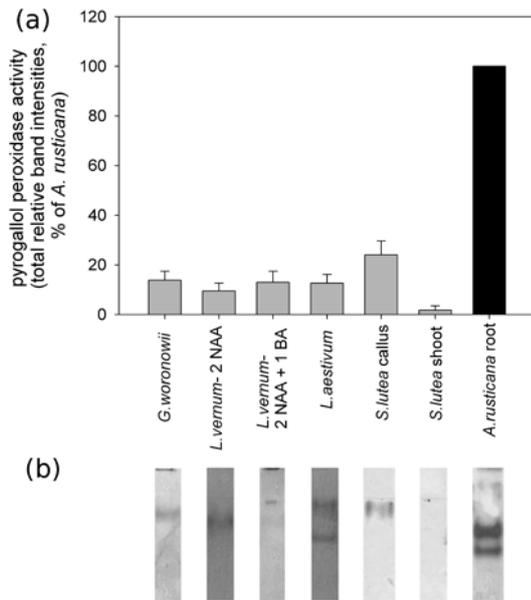
**Tab. 2** Summary of free radical scavenging capacities of Amaryllidaceae tissue cultures and organs of native plants involved in this study.

Sample	Total phenolics (GAE, mg g DW <sup>-1</sup> )	% inhibition of ABTS* cation <sup>b</sup>	Equivalent to mg AA per mL assay mixture	Equivalent to $\mu$ M AA / 0.5 mg extract used in the assay	ABTS* inhibition, TEAC ( $\mu$ M/0.5 mg extract used in the assay)
1a. <i>Galanthus woronowii</i> , micropropagated shoots, 2 NAA + 1 BA <sup>a</sup>	15.31 $\pm$ 1.7	77.36 $\pm$ 5.38	4.32 $\pm$ 0.82	25.00 $\pm$ 4.76	25.52 $\pm$ 3.08
1b. <i>G. woronowii</i> , native leaves	14.49 $\pm$ 4.15	66.33 $\pm$ 8.52	3.90 $\pm$ 0.65	22.73 $\pm$ 3.95	26.73 $\pm$ 4.48
1c. <i>G. woronowii</i> , native bulbs	12.00 $\pm$ 1.87	33.33 $\pm$ 5.50	1.96 $\pm$ 0.17	11.50 $\pm$ 0.92	14.00 $\pm$ 1.08
2a. <i>Leucojum vernum</i> , micropropagated plants, 2 NAA	6.17 $\pm$ 1.81	32.76 $\pm$ 1.61	1.06 $\pm$ 0.09	5.98 $\pm$ 0.50	10.93 $\pm$ 0.52
2b. <i>L. vernum</i> , micropropagated plants, 2 NAA + 1 BA	12.00 $\pm$ 2.7	59.2 $\pm$ 6.5	3.33 $\pm$ 0.50	19.26 $\pm$ 2.81	20.78 $\pm$ 3.16
2c. <i>L. vernum</i> , native leaves	22.71 $\pm$ 1.77	74.66 $\pm$ 5.6	4.36 $\pm$ 0.55	25.5 $\pm$ 3.25	26.13 $\pm$ 3.23
2d. <i>L. vernum</i> , native bulbs	4.63 $\pm$ 2.25	12.66 $\pm$ 1.72	0.72 $\pm$ 0.10	4.26 $\pm$ 0.69	5.76 $\pm$ 2.42
3a. <i>L. aestivum</i> , embryogenic callus derived whole plants, 2 NAA	12.33 $\pm$ 1.5	42.03 $\pm$ 6.24	1.71 $\pm$ 0.25	10.87 $\pm$ 1.46	14.68 $\pm$ 1.72
3b. <i>L. aestivum</i> , native leaves	15.93 $\pm$ 2.47	51.00 $\pm$ 8.03	2.96 $\pm$ 0.61	17.3 $\pm$ 2.29	18.83 $\pm$ 3.27
3c. <i>L. aestivum</i> , native bulbs	4.9 $\pm$ 1.52	12.33 $\pm$ 2.18	0.71 $\pm$ 0.11	4.23 $\pm$ 0.72	6.86 $\pm$ 1.63
4a. <i>Sternbergia lutea</i> , embryogenic callus, 2 NAA + 1 BA	15.93 $\pm$ 2.51	71.00 $\pm$ 6.13	4.13 $\pm$ 0.48	23.71 $\pm$ 2.72	24.81 $\pm$ 2.98
4b. <i>S. lutea</i> , somatic embryo derived regenerated shoots, 2 NAA + 1 BA	12.98 $\pm$ 2.03	73.66 $\pm$ 7.28	4.29 $\pm$ 0.60	24.58 $\pm$ 3.34	24.43 $\pm$ 4.17
4c. <i>S. lutea</i> , native leaves	23.7 $\pm$ 0.66	79.00 $\pm$ 4.04	4.90 $\pm$ 0.23	28.60 $\pm$ 1.44	26.60 $\pm$ 0.98
4d. <i>S. lutea</i> , native bulbs	18.4 $\pm$ 4.21	62.66 $\pm$ 8.87	3.70 $\pm$ 0.47	21.53 $\pm$ 2.71	22.26 $\pm$ 3.28

AA – ascorbic acid. <sup>a</sup> Numbers in the first column represent growth regulator concentrations in mg L<sup>-1</sup>. <sup>b</sup> Extracts contained 0.5 mg DW of plant material per mL assay mixture. Mean  $\pm$ SE values are presented;  $n \geq 10$

1 mg L<sup>-1</sup> BA had near 60% of ABTS\* scavenging capacity and relatively high TEAC and ascorbic acid equivalent values, thus can be considered of having high antioxidant properties as well (Tab. 2). Data including TEAC values showed low ABTS\* inhibition/scavenging values in cultures of *L. vernum* and *L. aestivum* grown in the presence of 2 mg L<sup>-1</sup> NAA (Tab. 1). Shoots of all native plants showed ABTS\* inhibition values similar to their corresponding tissue cultures and except *S. lutea*, native bulbs had low radical scavenging activities (Tab. 2).

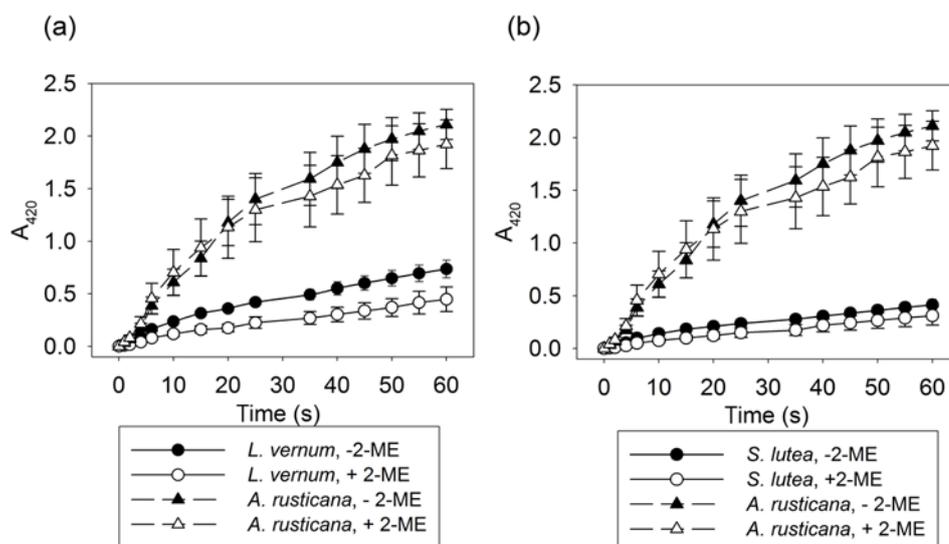
The protein contents of tissue culture extracts used for the assay of peroxidase activities were between 21.2  $\pm$ 4.35 (SE) and 30.9  $\pm$ 5.1  $\mu$ g for *G. woronowii*, *L. vernum*, and *L. aestivum* and 8.5  $\mu$ g (callus), 10.04  $\pm$ 3.18  $\mu$ g (shoots) for *S. lutea* per 10  $\mu$ L of samples loaded onto native activity gels. Thus, the protein contents were suitable for enzyme activity measurements. For pyrogallol peroxidase, *S. lutea* calli had the highest, yet still low activities, as compared to horseradish root extracts (Fig. 2a,b).



**Fig. 2** Pyrogallol peroxidase activities of Amaryllidaceae tissue cultures. **a** Quantification of activity gels (total relative band intensities /number of pixels per area) calculated with the aid of the CP Atlas software and expressed as percentage of the activity of horseradish root extracts (100%). Mean  $\pm$ SE values are plotted. Coloring of columns: black – high, grey – low activities. **b** Representative band patterns of samples.

In contrast, organs of native *S. lutea* plants had no detectable pyrogallol peroxidase activities on activity gels, and this was true for leaves and bulbs of all other native plants as well, having comparably low or no detectable activities relative to their corresponding in vitro cultures (data not shown). Spectrophotometric assay was performed with extracts of identical amounts of plant material as for activity gel electrophoresis (see ‘‘Material and methods’’) and showed that the absence of 2-ME in the extract induced only a slight activity increase, but this activity was still low as compared to horseradish root extracts for two representative cultures, *S. lutea* shoots and *L. vernum* whole plant, the latter grown in the presence of 2 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BA (Fig. 3a,b). Specific activities (mM oxidized pyrogallol min<sup>-1</sup> mg protein<sup>-1</sup>) showed that in the absence of 2-ME, *S. lutea* extracts had 28.29% of the activity of horseradish root extracts used as positive controls, while for *L. vernum*, this value was 19.45%. In the presence of 2-ME, *S. lutea* extracts had 25.86% of the activity of (2-ME treated) horseradish extracts and for *L. vernum* this value was 7.13%. For guaiacol peroxidase, activity gels for extracts containing 2-ME showed that all tissue cultures had low or non-detectable activity, as compared to horseradish extracts. Concerning organs of native plants, leaves and bulbs of *G. woronowii* had activities comparable to tissue cultures, while all other explants had very low or no guaiacol peroxidase activities (data not shown). Spectrophotometric assay showed more pronounced inhibition

of guaiacol peroxidase activities by 2-ME, than for pyrogallol peroxidase (data not shown). Specific activities (mM oxidized guaiacol min<sup>-1</sup> mg protein<sup>-1</sup>) in the absence of 2-ME were 32.84% of horseradish extracts for *S. lutea* and 60.31% for *L. vernum*. Interestingly, native activity gels showed differences in the pyrogallol isoenzyme patterns. For *G. woronowii* and *S. lutea*, the velocity of electrophoretic migration was lower than in *Leucojum* cultures (Fig. 2b). In addition, pyrogallol peroxidase activity pattern differed between cultures of *L. vernum* and *L. aestivum*; in the latter case, two isoenzymes could be detected (Fig. 2b).



**Fig. 3** Time courses of pyrogallol peroxidase activities (absorbance values at 420 nm indicate the degree of pyrogallol oxidation in the presence of H<sub>2</sub>O<sub>2</sub>) as shown by spectrophotometric assays of *L. vernum* (a) and *S. lutea* (b) enzyme extracts. The effects of 2-mercaptoethanol (2-ME) on enzyme activities are shown. Enzyme activities were compared to a positive control, root extracts of *Armoracia rusticana* (horseradish).

## Discussion

To our best knowledge, this study is the first report on establishment of efficient tissue cultures in *G. woronowii* and *S. lutea*. For *L. vernum* (by using different explants of commercial and native Polish origin), previous work reported the production of embryogenic calli from fruit explants [9]. The present study demonstrates that a micropropagation system, not involving the production of embryogenic calli, can also be established for this species. Besides our report, several studies reported the production of *L. aestivum* tissue cultures [7,26], but none of them involved the assay of antioxidant capacities (see below). All cultures showed high shoot / whole plant and (except *L. aestivum*) high bulblet regeneration frequencies (Tab. 1). In vitro produced bulbs/bulblets are generally highly suitable for ex vitro cultivation of tissue cultures (see [10] and references therein).

Several tissue cultures of Amaryllidaceae involve the growth regulators NAA, IAA as auxins and BA as a cytokinin [7,10,27,28]. Auxin/cytokinin combinations involving growth regulators mentioned are potent inducers of embryogenic callus formation and/or mass plant propagation in this family. The auxins mentioned above are more preferred than 2,4-D (2,4-dichlorophenoic acid), because they are not likely to induce somaclonal variability, therefore they are more suitable for germplasm preservation purposes [10]. We have already proved this for *G. nivalis* cultures, where AFLP analysis showed no genetic variation between native plants and tissue cultures grown in the presence of NAA and BA [10]. This is of particular importance when endangered/protected plants are used for mass propagation in vitro and those cultures are used for species/genotype conservation.

The general rule of morphogenetic effects of auxin/cytokinin combinations can be applied for *G. woronowii* and *L. aestivum* cultures, where auxins (a combination of NAA with IBA for *G. woronowii* and NAA alone for *L. aestivum*) applied without cytokinins promoted rooting, while a combination of NAA and BA induced the formation of shoots only (*L. aestivum*) or poor rooting (*G. woronowii*). IBA is known to promote rooting in many tissue cultures [29]. Since both auxins and cytokinins are required for the normal differentiation of both vegetative organs [11], it is likely that *G. woronowii* and *L. aestivum* cultures contain endogenous cytokinins necessary for both root and shoot production, thus adding of an auxin (e.g., NAA only) to the culture medium, together with the endogenous cytokinin promotes whole plant development. However, in the case of *L. vernum*, both the application of NAA as the sole growth regulator and of a combination of NAA and BA will promote rooting (Fig. 1b), indicating less endogenous cytokinin content than for *L. aestivum*.

What compounds could induce high radical scavenging capacities in several tissue culture extracts of Amaryllidaceae? Relatively high content of phenolics is generally correlated to high antioxidant capacities in medicinal plants [30]. *Galanthus reginae-olgae* native plants are good examples for Amaryllidaceae: high antioxidant activities were attributed for the probable high polyphenol content of aerial parts and bulbs [31]. A methylflavan with free radical scavenging properties was found in *Pancratium littorale* [32]. For *G. woronowii* and *S. lutea* cultures (and *S. lutea* native bulbs, the source of explants for tissue culture), high polyphenol content was accompanied with high ABTS\* scavenging capacity, while for our *L. aestivum* cultures, low free radical scavenging was detected even though polyphenol content of these samples was high (Tab. 2). Thus, polyphenolics have an essential contribution to the high antioxidant capacities of the former two cultures, while, probably because of different pattern of phenolic compounds, they are not sufficient for assuring high antioxidant capacity in *L. aestivum*. For *L. vernum* micropropagated cultures grown in the presence of NAA as the sole growth regulator, low phenolic content was accompanied with low ABTS\* scavenging capacity (Tab. 2), thus, as for cultures with high antioxidant capacities, here we detected the relationship between these two parameters.

To sum up results concerning phenolic contents and ABTS\* scavenging capacities, even though tissue culture conditions are known to alter secondary metabolite production and antioxidant capacities [30], they could preserve relatively high antioxidant activities of cultures involved in this study. It should be noted that our cultures involved the use of NAA, a synthetic auxin. The presence and nature of auxins are important for preserving high antioxidant capacities of plant tissue cultures [33]. In

addition, all cultures grown in the presence of both NAA and BA had high free radical capacities, which was not detected when NAA was used as the sole growth regulator (in case of the two *Leucojum* species), thus BA, a cytokinin, seems to increase ABTS\* scavenging capacity. At a careful examination of ABTS\* scavenging capacities (Tab. 2) we can conclude that although it was not possible to examine in vitro produced whole plants in all cases (*G. woronowii* and *S. lutea* produced only shoots, without root system), antioxidant activity depended on species (genotype) rather than the type of tissue or regenerant produced. Nota bene, all tissue cultures were initiated from the same tissue type: bulb scales, which can be an explanation to this. Different tissues within cultures of a single genotype (embryogenic callus vs. regenerated shoots in *S. lutea*) produce similar results when grown in the presence of the same PGR content (Tab. 2). This is true even though there are marked differences between different organs (leaves vs. bulbs) of native plants in this respect (Tab. 2).

Native *L. vernum* flowers and bulbs proved to have a relatively low antioxidant capacity when compared to other British medicinal plants [34]. In contrast, our native *L. vernum* leaves proved to have high free radical scavenging capacity (Tab. 2). The above studies indicate that secondary metabolite content is highly genotype- and organ-dependent even within a single species.

The study of antioxidant enzymes in plant tissue cultures has a double importance. (i) In vitro cultures, including calli and regenerants, are accompanied by stress reactions and different stages of callus development, cell proliferation or somatic embryogenesis, are accompanied with specific changes in isoenzyme (e.g., SOD isoenzyme activity) patterns [35]. Therefore, relevant studies give us physiological information on the levels of stress under standard in vitro conditions or at treatments with growth retardants [36]. (ii) The activity of antioxidant enzyme systems is relevant in pharmacological studies [37]. For several tissue cultures of this study, there were discrepancies between free radical scavenging capacity and peroxidase activities. The most prominent examples are the tissue culture systems of *G. woronowii* and *S. lutea*, where both embryogenic calli and regenerated shoots showed high free radical scavenging capacities, while the activities of enzymatic antioxidants (pyrogallol peroxidases) were low or not detectable. It should be noted, however, that ABTS\* cation inhibition assays show the ability to scavenge diverse oxygen radicals through non-enzymatic reactions [15,16], while peroxidase activities reflect a partially different phenomenon: the ability of enzymes to scavenge H<sub>2</sub>O<sub>2</sub> produced by non-enzymatic mechanisms or enzymatic radical scavengers like SOD (superoxide dismutase) [38]. Thus, for *G. woronowii* and *S. lutea*, probably low molecular weight compounds (polyphenolics) were primarily responsible for their high antioxidant capacities. *Sternbergia lutea* and *L. vernum* guaiacol peroxidase activities in extracts without 2-ME were relatively high. Meanwhile, for all our Amaryllidaceae tissue cultures, pyrogallol peroxidase activities were low as compared to root extracts of horseradish. Thus, none of cultures had high activities for both pyrogallol and guaiacol peroxidases.

In conclusion, we could establish successfully tissue cultures of four Amaryllidaceae species. These cultures are of potential importance for the germplasm preservation of endangered Amaryllidaceae species of Central Europe and whole Europe in general. Antioxidant capacities of these cultures were analyzed for the first time. Among them, cultures of *Leucojum aestivum* and *Sternbergia lutea* were embryogenic and somatic embryos could be converted efficiently to shoots or whole plantlets. In contrast, in vitro shoots or whole plants of *G. woronowii* and *L. vernum* could be produced by micropropagation that did not involve the production of somatic embryos. Cultures of *G. woronowii* and *S. lutea* were produced for the first time in this study and proved to be good free radical scavengers, preserving this feature of their corresponding wild-growing plants. Concerning enzymatic antioxidant capacities, none of cultures showed high activities of both pyrogallol and guaiacol peroxidases. Thus, we suggest that the high antioxidative effects of the two cultures mentioned above (*G. woronowii* and *S. lutea*) can be attributed primarily to non-enzymatic scavengers. The pharmacological use of compounds from these cultures is possible, thus further research on this topic is needed.

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