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Antioxidant potential of *Agrobacterium*-transformed and non-transformed *Physalis ixocarpa* plants grown *in vitro* and *ex vitro*

Potencjał antyoksydacyjny w transformowanych za pomocą *Agrobacterium* i nietransformowanych roślinach *Physalis ixocarpa* hodowanych *in vitro* i *ex vitro*

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Introduction: Oxidative stress is involved in pathogenesis of a number of chronic diseases hence is an increasing interest in plant-derived natural antioxidants with respect to their potential health benefits. Plants from the genus *Physalis* are particularly rich in secondary metabolites and show significant antioxidant potential. Recent development in transgenic research has opened new possibilities for enhanced production of secondary metabolites with plant cell and organ cultures. The hairy root-regenerated *Physalis ixocarpa* plants grown *in vitro* and *ex vitro* were compared to the non-transformed plants with respect to their antioxidant potential.

Material/Methods: The total antioxidant capacity (TAC), the contents of total phenols and ascorbate were evaluated in fruits, flowers, leaves and roots of *P. ixocarpa* using the ferric reducing antioxidant power assay (FRAP), the Folin-Ciocalteu method and the 2,2'-dipyridyl method, respectively.

Results/Discussion: The antioxidant profiles, in terms of TAC, ascorbate and phenols were organ-specific and depended on the culture conditions. Neither the total phenol content nor the ascorbate level appeared to determine the TAC of the studied plant extracts. The aqueous extracts exhibited lower antioxidant activities than the acetone ones indicating that lipophilic antioxidants made a major contribution to TAC of the plant tissues. *Agrobacterium* rhizogenes-mediated transformation changed the antioxidant status with respect to TAC, phenols and ascorbate and this effect was observed in the plants grown *in vitro* and *ex vitro*.

Key words: ***Physalis ixocarpa*** • **antioxidant capacity** • **ascorbate** • **phenols** • ***Agrobacterium*-mediated transformation**

Streszczenie

Wprowadzenie: Stres oksydacyjny jest zaangażowany w patogenezę wielu chorób przewlekłych, toteż wzrasta zainteresowanie naturalnymi antyoksydantami pochodzącymi z roślin w odniesieniu do ich potencjalnych korzyści zdrowotnych. Rośliny z rodzaju *Physalis* są bogate w metabolity wtórne i wykazują znaczny potencjał antyoksydacyjny. Postęp w badaniach nad transformacją genetyczną otworzył nowe możliwości w zwiększaniu produkcji metabolitów wtórnych w kulturach komórek i organów. Zregenerowane z korzeni transformowanych rośliny *Physalis ixocarpa*

porównano z roślinami nietransformowanymi hodowanymi *in vitro* i *ex vitro* pod kątem potencjału antyoksydacyjnego.

Materiał/Metody: Całkowity potencjał antyoksydacyjny (TAC), całkowitą zawartość fenoli i askorbinianu mierzono w owocach, kwiatach, liściach i korzeniach *P. ixocarpa* odpowiednio, na zasadzie redukcji jonów żelaza (FRAP), z odczynnikiem Folin-Ciocalteu i metodą z 2,2'-dipyrydylem.

Wyniki/Dyskusja: Profil antyoksydacyjny wyznaczony na podstawie TAC, arkorbinian i fenole zależał od organu i warunków hodowli. Wydaje się, że całkowita zawartość fenoli, a nie poziom askorbinianu określają TAC w badanych ekstraktach. Wodne ekstrakty wykazywały mniejszą aktywność antyoksydacyjną w porównaniu do ekstraktów acetonowych, co wskazuje, że lipofilne antyoksydanty mają większy udział w całkowitym potencjale antyoksydacyjnym w tkankach roślin. Transformacja za pośrednictwem *Agrobacterium* zmieniała status antyoksydacyjny w odniesieniu do TAC, fenoli i askorbinianu, co było widoczne zarówno w roślinach hodowanych *in vitro*, jak i *ex vitro*.

Słowa kluczowe: ***Physalis ixocarpa*** **• całkowity potencjał antyoksydacyjny • askorbinian • fenole • transformacja za pośrednictwem *Agrobacterium***

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Introduction

Plants from the genus *Physalis* (family *Solanaceae*), native to warm and subtropical regions of Middle and South Americas, are particularly rich in secondary metabolites. Phytochemical analyses showed that they contain witanolides, physalins, calystegines, tropane and nortropane alkaloids [32,22]. *Physalis* species have also shown significant antioxidant potential [6,24]. Due to the large biological activities of these compounds, *Physalis* plants were used for centuries as medicinal herbs in the treatment of urinary and skin diseases, gonorrhea, ulcers, sores and as a vermicidal drug, and recent studies have confirmed their therapeutic properties [31,15]. *Physalis* spp. extracts have been also reported to possess anticancer activities [16,6].

Out of the secondary metabolites found in the genus *Physalis*, the physalins have been most extensively screened for their biological effects [2,16,8] whereas antioxidants have been assessed less intensely. However, as oxidative stress is involved in pathogenesis of a number of chronic diseases including cancer, cardiovascular and neurodegenerative disorders [23,26], there is an increasing interest in plant-derived natural antioxidants with respect to their potential health benefits. In plants, ascorbate and phenolic compounds contribute mainly to the total antioxidant potential of plant extracts and may be the basis for their biological activity.

Recent development in transgenic research has opened new possibilities for enhanced production of secondary metabolites with plant cell and organ cultures. It has been found that *Agrobacterium*-mediated transformation affected the content of secondary metabolites as rol genes act as potent activators of secondary metabolism [5]. Thus the use of transformed root cultures (hairy roots) which are established by transformation with *A. rhizogenes* is a promising approach to intensify the production of secondary metabolites. Furthermore, differentiated plants regenerated from the hairy roots can be also used for the production of bioactive metabolites in good yields. However, there are only a few reports on secondary metabolite production in hairy root-regenerated medicinal plants [27]. Additionally, most of the data did not refer to the plant growth stage and organ specificity of the metabolite profiles.

The aim of this study was to compare the hairy root-regenerated *Physalis ixocarpa* plants grown *in vitro* and *ex vitro* to the non-transformed plants with respect to their antioxidant potential in terms of total antioxidant capacity (TAC) and the contents of total phenols and ascorbate.

Materials and Methods

Plant material

Non-transformed *Physalis ixocarpa* Brot. ex Hornem (syn. *Physalis philadelphica* Lam.) plants were obtained from *in vitro* germinated seeds and cultured *in vitro* on solidified Murashige and Skoog [19] basal medium with 0.7% (w/v) agar and 3% sucrose (w/v), supplemented with 5 μM kinetin and 1 μM 6-benzyladenine for shoot induction and with 1 μM 1-naphthaleneacetic acid for rooting. Cultures were grown under 16 h/8 h photoperiod (40 μmol m^{-2} s^{-1} cool fluorescent light, Philips 40W) at 23±2°C. Transformed plants were obtained by regeneration from hairy roots transformed with *Agrobacterium rhizogenes* ATCC 15834 and

Table 1. Total antioxidant capacity (TAC) of water-soluble (TACw) and acetone-soluble (TACa) antioxidants in hairy root cultures and in roots and leaves of non-transformed and *Agrobacterium rhizogenes*-transformed *Physalis ixocarpa* plants cultured *in vitro*

Source	TAA (Trolox equivalents g^{-1} FW)			
	TACw	TACa	Total	TACw/TACa
Hairy roots	0.238[a]±0.033	0.545±0.087	0.783±0.109	0.44
Roots of non-transformed plants	0.510±0.049	0.711±0.035	1.221±0.109	0.72
Leaves of non-transformed plants	0.464±0.045	2.170±0.379	2.634±0.477	0.21
Roots of transformed plants	0.264±0.036	1.489±0.274	1.753±0.286	0.18
Leaves of transformed plants	1.620±0.356	5.810±1.174	7.430±1.471	0.28

[a] Data are expressed as the mean ± standard deviation (n=3).

cultured *in vitro* under the same conditions as described above. For acclimatization to *ex vitro* conditions at day 28 non-transformed and transformed plants were washed in sterile distilled water to remove traces of MS medium and transferred to pots containing perlite under the growth chamber conditions (23±2°C, 16 h/8 h photoperiod, 70% humidity and 40 μmol m^{-2} s^{-1} light intensity) and covered for 7 days with glass. After 14 days the plants were transferred to pots filled with soil and grown under the controlled growth chamber conditions as described above. Plants cultivated *in vitro* were used for experiments at the age of 4 weeks and plants grown *ex vitro* were analyzed 8–10 weeks after having been transferred to soil.

Total antioxidant capacity

For extraction of water-soluble and acetone-soluble antioxidants 500 mg of fresh plant material (roots, leaves, flowers and fruits) was homogenized (1:10) in 0.1 M phosphate buffer (pH 7.4) and acetone, respectively. Then the mixtures were centrifuged and supernatants were used for measurements of the total antioxidant capacity of water-soluble (TACw) and acetone-soluble antioxidants (TACa). TAC was measured by using a modified FRAP (Ferric Reducing Ability of Plasma) assay [3]. This method depends upon the reduction of ferric 2,4,6-tripyridyltriazine (Fe (III)-TPTZ) complex to the ferrous 2,4,6-tripyridyltrazine (Fe (II)-TPTZ) by a reductant at low pH, monitored at 593 nm. Briefly, the FRAP reagent was prepared fresh daily from 300 mM acetate buffer at pH 3.6, 10 mM TPTZ (2,4,6-tripyridyltriazine) solution in 40 mM HCl and 20 mM $FeCl_3 \times 6\ H_2O$ solution in proportion of 10:1:1 (v/v), respectively. Plant extracts (50 μl) were allowed to react with the FRAP reagent for 30 min in darkness. The absorbance of the reaction mixture was than recorded at 593 nm. The antioxidant capacities of the extracts were expressed as mg Trolox equivalents per gram of fresh plant material (mg g^{-1} FW).

Determination of total phenols

For the determination of total phenols plant material (0.5 g FW) was homogenized in 5 cm^3 ice-cold 80% aqueous methanol. Total phenol content was measured by a modified Folin-Ciocalteu assay [29]. Briefly, methanolic extract (0.1 cm^{-3}) was mixed with 3.8 cm^{-3} distilled water, 0.1 cm^{-3} Folin-Ciocalteu reagent and 1 cm^{-3} 10% Na_2CO_3. The mixture was incubated for 1 h at room temperature and then the absorbance at 725 nm was measured. Calibration curve was prepared with chlorogenic acid and total phenol content was given as chlorogenic acid equivalents in μmol g^{-1} FW.

Determination of ascorbate content

For the determination of ascorbate content plant material (0.5 g FW) was homogenized in 5 cm^3 ice-cold 5% trichloroacetic acid. Ascorbate was determined spectrophotometrically by the method described by Kampfenkel et al. [13]. Total ascorbate was estimated after reduction of dehydroascorbate (DHA) to reduced ascorbate (AA) with dithiothreitol. The concentration of ascorbate (μmol g^{-1} FW) was determined using a calibration curve for AA as a standard. Redox ratio for ascorbate was calculated as AA/DHA.

Statistical analysis

The results presented are the means of three independent experiments. Sample variability is given as the standard deviation of mean.

Results and Discussion

Hairy root-regenerated plants of *P. ixocarpa* grown *in vitro* showed phenotype features typical for plants transformed with *A. rhizogenes*, i.e. reduced apical dominance, short internodes and wrinkled leaves. The hairy root-regenerated plants grown *ex vitro* showed normal morphology, however the plant height and leaf area were reduced when compared with the non-transformed culture-derived plants (data not shown).

It is well known that in plants the profiles of biologically active compounds with potential health benefits, e.g. antioxidants, depend on genetic, developmental and environmental factors [14,34]. These aspects need to be also considered with respect to antioxidant production in plants *in vitro*-cultures [17]. In our study the antioxidant tests based on a FRAP assay revealed that the differentiated *A. rhizogenes*-transformed plants regenerated from hairy roots and cultured *in vitro* exhibited the highest TAC, with 1.753 TE g^{-1} FW for roots and 7.43 TE g^{-1} FW for leaves. In the non-transformed plants grown *in vitro* the TAC of the root and leaf extracts were 39% and 64% lower, respectively. The lowest TAC (0.783 TE g^{-1} FW) was found in the hairy roots (Table 1). In general, the aqueous extracts exhibited lower antioxidant activities than the acetone ones indicating that under *in vitro* conditions the lipophilic antioxidants made

Table 2. Total phenol, reduced (AA), oxidized (DHA) and total ascorbate contents and ascorbate redox ratio (AA/DHA) in hairy root cultures and in roots and leaves of non-transformed and *Agrobacterium rhizogenes*-transformed *Physalis ixocarpa* plants cultured *in vitro*

Source	Phenols (μmol g^{-1} FW)	Ascorbate (μmol g^{-1} FW)			
		AA	DHA	Total	AA/DHA
Hairy roots	5.550[a]±0.999	0.972±0.204	0.308±0.046	1.280±0.243	3.15
Roots of non-transformed plants	4.760±0.776	0.921±0.278	0.733±0.121	1.654±0.459	1.26
Leaves of non-transformed plants	10.080±1.210	4.955±1.238	0.772±0.092	5.727±1.317	6.86
Roots of transformed plants	3.398±0.911	0.938±0.201	0.228±0.038	1.166±0.256	4.11
Leaves of transformed plants	8.007±1.436	7.200±1.289	0.429±0.078	7.629±1.396	16.78

[a] Data are expressed as the mean ± standard deviation (n=3).

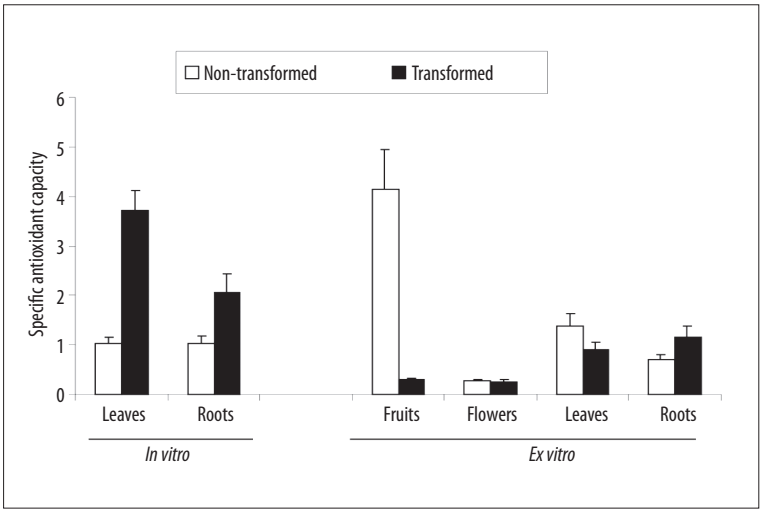

Fig. 1. Specific antioxidant capacity in organs of non-transformed and *Agrobacterium rhizogenes*-transformed *Physalis ixocarpa* plants cultured *in vitro* and grown *ex vitro*. Data are expressed as the mean ± standard deviation (n=3). The specific antioxidant capacity was defined as the ratio of total antioxidant capacity over total phenolics (Trolox mass equivalents per mass of chlorogenic acid)

a major contribution to TAC of the plant tissues. The lowest TACw/TACa index was found in the roots of transformed plants (Table 1).

The phenolic content of plants greatly contributes to their antioxidant potential and many studies have demonstrated the radical scavenging properties of plant phenolic compounds and confirmed the relationship between phenolic compounds and antioxidant activity [35]. However, some authors could not found such a relationship [18]. In this study no strong correlation between TAC and total phenol concentration was observed in cultures *in vitro*. The rank of total phenol content was as follows: leaves of non-transformed plants > leaves of transformed plants > roots of non-transformed plants > hairy roots > roots of transformed plants (Table 2) and it was not similar to the TAC rank (Table 1). It is likely that not all phenols are reducing agents active in the FRAP assay. Moreover, this correlation does not consider the qualitative and quantitative differences in the phenolic profiles among plant tissues. It has been proposed that the specific antioxidant capacity (TAC expressed on phenolics basis), could be a reliable indicator of the effectiveness of neutralization of free radicals by the specific mixture of phenolic compounds present in the tissue [11]. In our study transformation increased the specific antioxidant capacity in plants grown *in vitro* indicating the presence of phenolics with higher antioxidant capacity to stabilize free radicals when compared to the phenolic profiles present in the tissues of non-transformed plants (Fig. 1). The phenol content was organ-dependent, as its concentration in leaves was markedly higher than in roots and hairy roots (Table 2). A similar relationship was also found for ascorbate (Table 2). The latter corresponded to the fact that the highest level of ascorbate synthesis takes place in the leaves [30], and ascorbate levels in roots are usually low compared to leaves [9]. In our study, under *in vitro* conditions, the highest ascorbate content of 7.629 μmol g^{-1} FW was assayed for the leaves of transformed plants while their roots had the lowest ascorbate concentration of 1.166 μmol g^{-1} FW (Table 2).

Ascorbic acid is one of the most abundant and effective antioxidants in plant tissues. It is involved directly in eliminating reactive oxygen species, regenerating vitamin E in plants and it also participates in cell metabolism and signaling [20]. The antioxidant potential of ascorbate depends not only on its content but also on the redox status of its pool. Our results indicated that the ascorbate-dependent redox environment was kept reduced as the total ascorbate pool was dominated by AA (Table 2). However, the increased AA content and diminished DHA concentration in the leaves and roots of the transformed plants as compared with the non-transformed ones resulted in higher ascorbate redox state expressed as the AA/DHA ratio (Table 2). In the leaves of transformed plants the high ascorbate level corresponded to the increased activity of the water-soluble antioxidants, whereas in the non-transformed plants it did not (Tables 1, 2). These results confirmed that under *in*

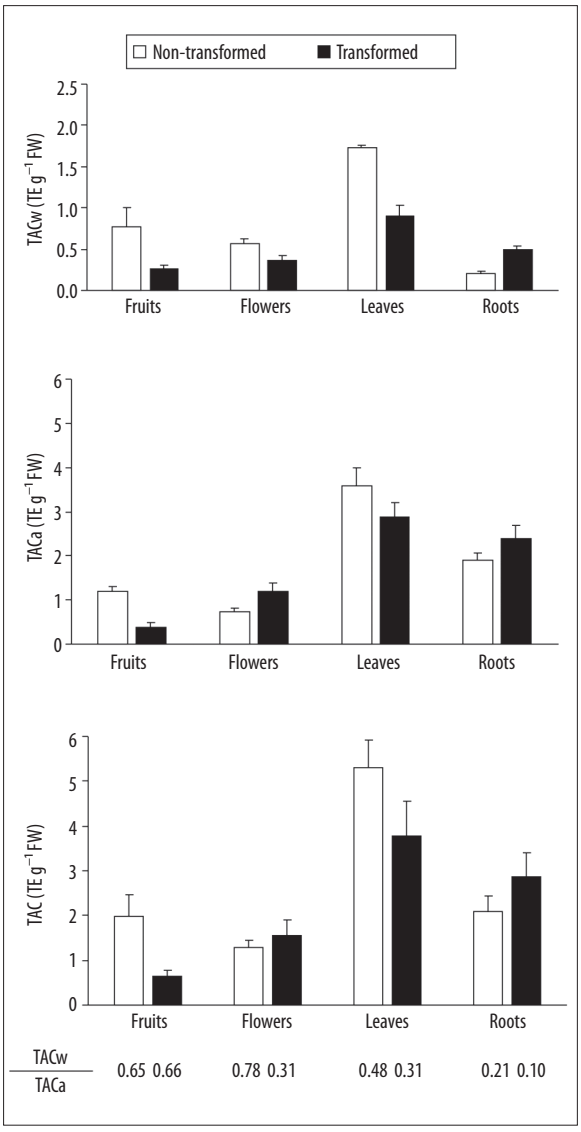

Fig. 2. Total antioxidant capacity (TAC) of water-soluble (TACw) and acetone-soluble (TACa) antioxidants in organs of non-transformed and *Agrobacterium rhizogenes*-transformed *Physalis ixocarpa* plants grown *ex vitro*. Data are expressed as the mean ± standard deviation (n=3)

vitro conditions leaves of the transformed and non-transformed plants differed in their antioxidant profiles. As genetic transformation of different Physalis species for the production of secondary metabolites has been reported [1,2] these data might be important for the *in vitro* biosynthesis of antioxidants from *Physalis* cultures.

In *Physalis* grown *ex vitro* the antioxidant characteristics varied between the transformed and non-transformed plants, but mostly in relation to organs. Leaves had the highest TAC of 5.314 and 3.764 TE g^{-1} FW for non-transformed and transformed plants grown *ex vitro*, respectively (Fig. 2). The lowest values of TAC, 1.289 and 0.654 TE g^{-1} FW, were determined in flowers of the non-transformed plants and fruit extracts of the transformed ones. In flowers and roots, however the TAC values were higher in the transformed plants (Fig. 2). Similar relationships between the transformed and non-transformed plants were

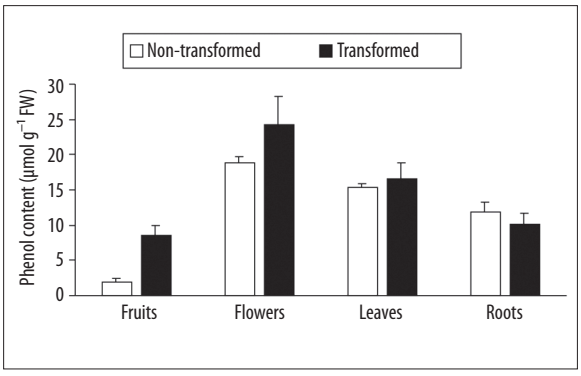

Fig. 3. Total phenols content in organs of non-transformed and *Agrobacterium rhizogenes*-transformed *Physalis ixocarpa* plants grown *ex vitro*. Data are expressed as the mean ± standard deviation (n=3)

found with respect to the antioxidant capacities of water- and acetone-extractable antioxidants, except the TACw of flower extracts which was higher in the non-transformed plants (Fig. 2). The lower TAC of the leaf and fruit extracts in *A. rhizogenes*-transformed plants could be attributed to the transformation-induced changes in the antioxidant profile. Similarly to the proportions described for *in vitro* cultures, the acetone extracts exhibited higher antioxidant capacity than the aqueous ones and the lowest ratio of TACw to TACa was found in the roots (Fig. 2). The highest concentration of phenolic compounds was found in the flowers and the lowest in the fruits (Fig. 3). In the generative organs (fruits, flowers) the levels of phenolics were higher in the transformed plants whereas in the vegetative organs (leaves, roots) their contents were similar to those in the non-transformed ones (Fig. 3). As it has been found that high rates of biomass production had negative effects on the respective production of secondary metabolites [7], the lower content of phenols in the fruits and flowers in the non-transformed plants could be attributed to more intensive growth of these organs when compared to the transformed ones (data not shown).

Phenols are often recognized as the compounds with major relevance in the TAC of fruits and vegetables [21,35]. In *P. peruviana*, elagic acid was suggested to be the major component contributing to the antioxidant activity of the whole-plant aqueous extracts [6]. Taking into account that the fruits of *P. ixocarpa* (tomatillos) are popular dietary products traditionally used as ingredients in sauces in some countries in Latin America [4,32], it is worth noting that in fruits from *A. rhizogenes*-transformed plants the content of total phenols was 4.5-fold higher than in the respective organs of non-transformed plants (Fig. 3). However, the increased level of phenols did not correspond to TAC (Fig. 2) and to specific antioxidant capacity (Fig. 1) indicating the importance of other antioxidant constituents in the fruits of transformed plants, or the negative effects of changes in the phenolic pool on its antioxidant activity. The structure--activity relationship studies have revealed that the degree of glycosylation and hydroxylation affects the antioxidant properties of phenolic compounds [10]. Thus the antioxidant capacity of a given plant extract cannot be predicted on the basis of the total phenolic content, as was observed also in the present study. Similar results have been reported for a large number of plant material extracts of Finnish

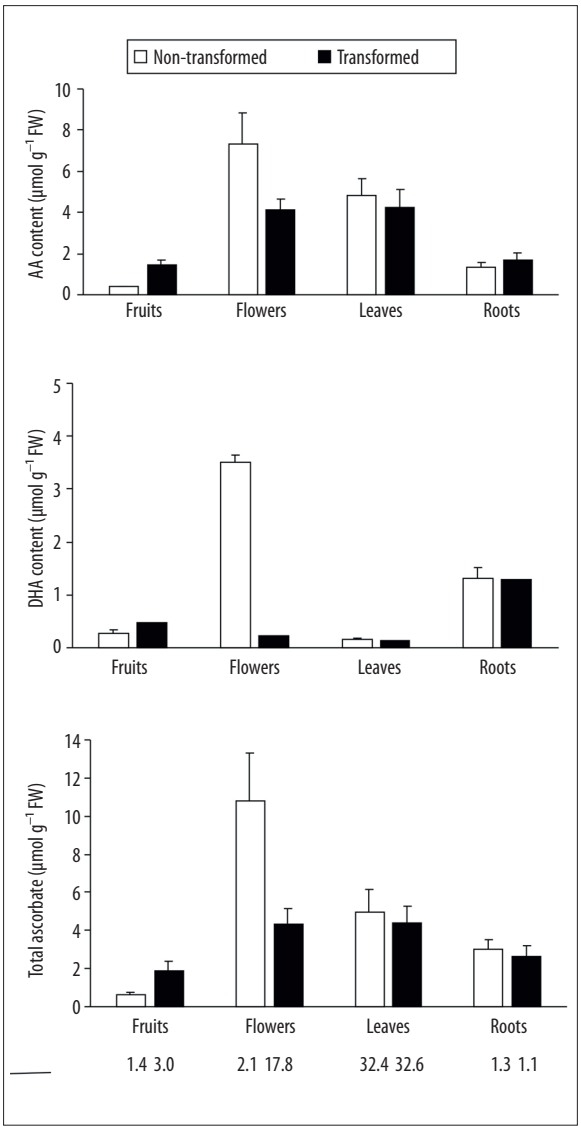

Fig. 4. Concentration of reduced (AA), oxidized (DHA) and total ascorbate contents and ascorbate redox ratio (AA/DHA) in organs of non-transformed and *Agrobacterium rhizogenes*-transformed *Physalis ixocarpa* plants grown *ex vitro*. Data are expressed as the mean ± standard deviation (n=3)

origin evaluated with respect to their total phenolic content and antioxidant activity [12].

In Physalis plants grown *ex vitro* the content and redox ratio of ascorbate was organ-specific. The leaves of both transformed and non-transformed plants showed high AA content (4.84 µmol g^{-1} FW and 4.24 µmol g^{-1} FW, respectively) and the highest AA/DHA redox ratio (Fig. 4). The generally high level of ascorbate accumulation in plant leaves can be explained by its constitutive role in photosynthesis [20]. Intriguingly, we observed that the flowers of non-transformed plants contained approximately 2 times the levels of AA and total ascorbate found in the leaves and the flowers of transformed plants maintained approximately the same content of ascorbate as the leaves (Fig. 4). Moreover, the flowers of transformed and non-transformed plants showed differential accumulation of DHA (Fig. 4) and thus differed significantly with respect to their redox ratio of ascorbate (17.8 *versus* 2.1). Ascorbate was the least abundant in the fruits followed by roots (Fig. 4). Transformation caused marginal difference in the ascorbate level in the roots. In the fruits, however the contents of AA and DHA were markedly higher in the transformed than in non-transformed plants. As an opposite relationship was found with respect to TACw, these data could indicate that in the fruits of transformed plants ascorbate did not account for the activity of water-soluble antioxidants. The contribution of AA to total antioxidant power in aqueous extracts of popular fruits ranged from 1 to 50% [33]. Taking into account the literature data revealing high variability of AA content in fruits [25,33], its level determined in Physalis fruits appeared to fall within the lower range. Thus, tomatillos cannot be recommended as a rich dietary source of vitamin C.

Conclusions

In conclusion, our study provided the first indication of differential TAC and accumulation of phenols and ascorbate in the organs of *A. rhizogenes*-transformed and non-transformed *Physalis* plants grown *in vitro* and *ex vitro*. The antioxidant profiles, as far as TAC, AA and phenols were concerned, were organ-specific and depended on the *in vitro* and *ex vitro* culture conditions. Neither the total phenol content nor the ascorbate level appeared to determine the TAC of the studied plant extracts. Transformation changed the antioxidant status in terms of TAC, phenols and ascorbate and this effect was observed in the plants grown *in vitro* and *ex vitro*. As phytochemicals of medicinal plants have been receiving increased interest, this preliminary study contributes to the characteristics of secondary metabolites with antioxidant activity in the genus *Physalis*.

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