Glycoalkaloid Profile in Potato Haploids Derived from *Solanum tuberosum*–*S. bulbocastanum* Somatic Hybrids

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Cultivated and wild potato species synthesize a wide variety of steroidal glycoalkaloids (GA) that may affect either human health or biotic stress resistance. Therefore, GA composition must be a major criterion in the evaluation of breeding products when species genomes are merged and/or manipulated. This work reports the results of GA analysis performed on unique haploid ($2n=2x=24$) plants obtained from tetraploid ($2n=4x=48$) *Solanum bulbocastanum*–*S. tuberosum* hybrids through *in vitro* anther culture. Glycoalkaloids were extracted from tubers and analyzed by HPLC. Haploids generally showed the occurrence of parental GA. However, in several cases loss of parental GA and gain of new GA lacking in the parents was observed. It may be hypothesized that new GA profiles of our haploids is the result of either genetic recombination or combinatorial biochemistry events. To highlight differences between haploids and parents, soluble proteins and antioxidant activities were also determined. Both were always higher in haploids compared to their parents. The nature of the newly formed GAs will be further investigated, because they may represent new metabolites that can be used against pest and diseases, or are useful for human health.

Introduction. – Glycoalkaloids (GA) are typical secondary metabolites occurring in a wide range of plants. They may have both adverse and beneficial effects. High GA concentrations impart a bitter taste and may be toxic for humans. Indeed, at the cellular level they cause membrane disruption and inhibit acetyl cholinesterase activity [1]. Other possible effects include teratogenicity, embryotoxicity, and genotoxicity [2]. Not only the amount, but also the type of GA is an important aspect determining toxicity. *Roddick and Rijnenberg* [3] demonstrated that α-solanine and α-chaconine act synergically in cell disruption effects. In contrast, some GAs can also have positive effects on plant growth and human diet. As recently outlined by *Friedman* [4], they may provide plant protection from damage by insects and fungi, and show anti-inflammatory and anticancer effects in humans. Species belonging to the *Solanaceae* family, including the cultivated potato *Solanum tuberosum* and its wild relatives, synthesize at least 80 structurally different GAs [5]. *S. tuberosum* usually contains α-solanine and α-chaconine, which are triose glycosides of solanidine. Their ratio in potato plants depends on the genotype, tissue, and growing and storage conditions [6]. Wide variability exists in the amounts and types of GA in wild potato species. *Deahl et al.* [7] reported twelve major GAs in the leaves of *ca.* 70 potato species. Solamargine and solasonine have been reported in more than 100 potato species [1]. In contrast, leptinine I and II, and leptine I and II occur only in *S. chacoense* [8]. Toxicity of GAs

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has necessitated regulations limiting their amounts in cultivated potatoes to a level that is expected to cause negligible risks to humans (< 200 mg/g tuber fresh weight).

Potato biodiversity represents a unique and valuable source of useful genes to improve agronomic, resistance, and quality characteristics of the cultivated potato. Therefore, it is largely used in breeding programs based on either sexual/somatic interspecific hybridization or on direct gene transfer through genetic transformation [9]. Among wild Solanums, the Mexican *S. bulbocastanum* is a very interesting species, especially for its resistance against *Phytophthora infestans*, the most dangerous potato pathogen. Due to post-zygotic incompatibility barriers, sexual crosses between *S. bulbocastanum* and *S. tuberosum* cannot be performed to introgress noteworthy genes into the cultivated gene pool. In contrast, somatic hybridization through protoplast fusion is a valid strategy to produce interspecific hybrids [10]. In the frame of a breeding program aimed at efficiently exploit this wild species, we have produced tetraploid ($2n = 4x = 48$) *S. bulbocastanum* (+) *S. tuberosum* somatic hybrids. They have been characterized for phenotypic traits of interest and also for their GA patterns [11][12]. Determination of GAs in genotypes containing the *S. bulbocastanum* genome is considered very important, since several GAs have been identified in tubers and leaves of this species. A recent quantitative analysis of GA content in wild Solanums [13] revealed that *S. bulbocastanum* shows the highest concentration of tuber GA. To continue breeding efforts, another culture has been employed *in vitro* to reduce the ploidy level of our *S. bulbocastanum* (+) *S. tuberosum* hybrids through production of haploid ($2n = 2x = 24$) plants [12]. Haploids have a gametic chromosome number. Therefore, they represent unique genetic materials for basic research and applied breeding [14][15]. This article aimed to compare by HPLC the GA distribution and patterns in tubers of *S. tuberosum–S. bulbocastanum* haploids relative to those of the somatic hybrids from which they are derived. In addition, to highlight differences between haploids and parents, soluble proteins and antioxidant activities were also determined.

Results and Discussion. – In this study, unique *S. bulbocastanum–S. tuberosum* haploid plants were investigated for GA profile. These materials represent a precious breeding resource to improve traits lacking/defective in the cultivated potato. Since GA content is genetically controlled, GA composition should be a major criterion for the development and release of new potato varieties [16]. For GA analysis, plant materials were grown in a temperature-controlled greenhouse, under the same microclimatic conditions. Large differences were found in the amount of dried tubers and the basic BuOH extracts obtained for each genotype (*Table 1*). Weight differences observed in dried tubers (range 2.6–4.2 g/20 g fresh tuber) can be attributed to differences in maturity of tubers collected and, therefore, in water content. The basic extract, containing essentially GAs, was higher in *S. bulbocastanum–S. tuberosum* haploids (63.6 mg/g dried tuber) than in 'Spunta' (17.6 mg/g dried tuber). This is probably due to the fact that, with respect to several traits, the combination of two different genomes may result in the phenotypic superiority of hybrid genotypes over their parents. This phenomenon, genetically known as heterosis, is well-documented in plants [17].

To analyze the GA content of the basic organic extract of our haploid genotypes, a HPLC method was applied. This method was previously used to determine the GA

Table 1. *Dried Tuber Content and Corresponding Basic Organic Extract Obtained from Haploid Genotypes Extracted from* Solanum bulbocastanum (+) S. tuberosum *Somatic Hybrids through Anther Culture. S. tuberosum* control 'Spunta' is also reported.

Haploid	Parental somatic hybrid	Dried tuber [g/20 g fresh tuber]	Extract [mg/g dried tuber]
AS5A1	HF 5A	3.9	61.6
AS6A1	HF 6A	3.5	42.3
AS6A2	HF 6A	3.2	57.4
AS6A4	HF 6A	2.6	68.8
AS6A5	HF 6A	4.2	41.0
AS6A6	HF 6A	3.4	105.3
AS6E1	HF 6E	3.0	86.9
AS9I2	HF 9I	2.8	57.8
AS9AP1	HF 9AP	3.4	47.1
AS9AP2	HF 9AP	3.1	67.9
'Spunta'		3.7	17.6

profile of the parental hybrids of our haploids [11] and was derived from [1]. It should be also pointed out that the GA nature of the unidentified molecules associated to peaks present in the HPLC chromatographic profile was ascertained by chemical degradation and spectroscopic analysis. HPLC Analyses were carried out using α-solanine and α-chaconine, the main GAs of *S. tuberosum*, as reference compounds. As expected, the analysis of the chromatographic profile of 'Spunta' showed only α-solanine (S) and α-chaconine (C) peaks (not shown). The HPLC profile of the organic extract of five anther-derived haploids of somatic hybrid HF6A is shown in the *Figure*. In describing the GA profile, we used the same codes reported in [11]. Compared to their parent, three haploids (AS6A1, AS6A2, and AS6A4) lost the peak labelled *5* (t_R 10.39 min). Loss of this peak in some HF6A haploids suggests that this GA may be unstable in that, following meiosis, genes controlling the formation of sugar moiety and aglycone of this GA may easily segregate. The *Figure* also highlights that, in three haploids (AS6A4, AS6A5, and AS6A6), one new peak (labelled *8*, t_R 7.80 min) was observed. Since haploids were produced from meiosis-derived microspores, it may be hypothesized that the new GA is the result of genetic recombination. Alternatively, epigenetic effects or combinatorial biochemistry events are also possible [18]. They occur when genes involved in the biosynthesis of a specific compound are introgressed into the genome of another species producing related molecules of potential interest. *Laurila et al.* [19] reported that *S. tuberosum–S. brevidens* somatic hybrids produced not only parental GA but also demissidine, which has never been detected in the parents, and four unidentified compounds. The nature of the newly formed GA we found in haploid plants should be further investigated not only because it may be hazardous for humans, but also because it may be used against pests and diseases or useful for human health. Loss of parental GA and gain of new GA lacking in the parents have been observed also in all other haploids (*Table 2*). AS5A1 lost peak *4* compared to its parent; AS6E1 displayed one new peak (*5*) and lost α-solanine; AS9I2 showed one new peak labelled *9* (t_R 12.50 min); AS9AP1 and AS9AP2 lost peak *6* and gained peak *5*. Even in a small sample size, these results demonstrate that scaling down the ploidy level of *S. bulbocastanum–S. tuberosum* hybrids may drastically change the

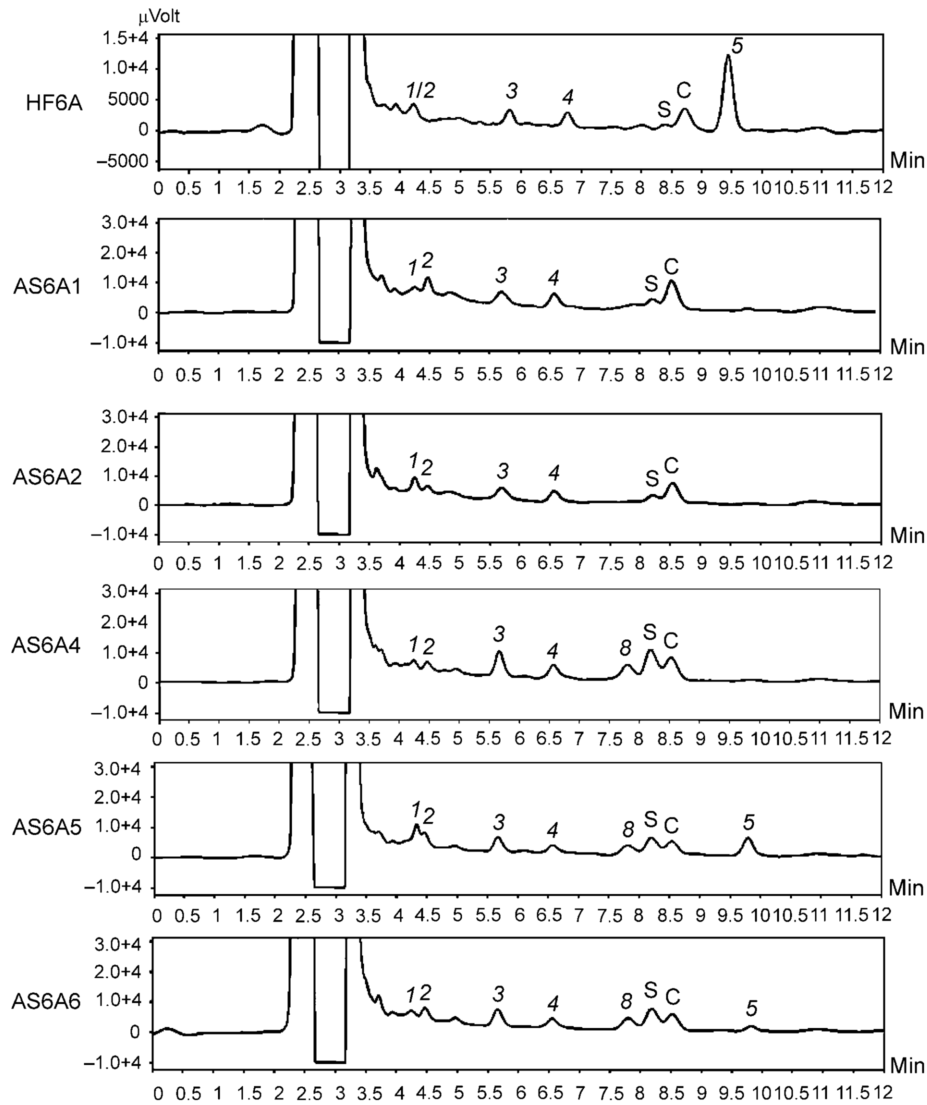

Figure. *HPLC Chromatograms of the basic extract obtained from a tetraploid somatic hybrid* (HF6A, $2n=4x=48$) *and its anther-derived haploids* (AS6A1, A2, A4, A5, A6, $2n=2x=24$). Peaks *1–8*, unidentified glycoalkaloids; S, solamine; C, chacomine.

GA profile of newly produced genetic materials. Our findings also suggested that some GAs (*e.g.*, that corresponding to peak *5*) may be lost more rapidly than others, with either possible advantages (if it is toxic) or disadvantages (if important for pest control) in terms of breeding efforts. It can be hypothesized that the pathway of this GA is monogenic, or that there might be genes that can disrupt the pathway leading to its production [20]. It is well-known that GAs in potato are genetically controlled [5].

Table 2. *HPLC Qualitative Analysis* (+: presence; −: absence) *of Glycoalkaloid Content in Tubers of Haploids Extracted from* Solanum bulbocastanum (+) S. tuberosum *Somatic Hybrids and of* S. tuberosum *Control Spunta.* For comparison, in parenthesis, the presence/absence of tuber glycoalkaloids in parental somatic hybrids is given (from [11]).

Haploid	Parental somatic hybrid	Identified glycoalkaloids		Unidentified glycoalkaloids (peak #)								
		α-Chaconine 8.45[a])	α-Solanine 8.13	*1* 4.33	*2* 4.55	*3* 5.55	*4* 6.23	*5* 10.39	*6* 5.65	*7* 8.84	*8* 7.80	*9* 12.50
AS5A1	HF5A	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	− (+)	− (−)	− (−)	− (−)	− (−)	− (−)
AS6A1	HF6A	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	− (+)	− (−)	− (−)	− (−)	− (−)
AS6A2	HF6A	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	− (+)	− (−)	− (−)	− (−)	− (−)
AS6A4	HF6A	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	− (+)	− (−)	− (−)	+ (−)	− (−)
AS6A5	HF6A	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	− (−)	− (−)	+ (−)	− (−)
AS6A6	HF6A	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	− (−)	− (−)	+ (−)	− (−)
AS6E1	HF6E	+ (+)	− (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (−)	− (−)	− (−)	− (−)	− (−)
AS9I2	HF9I	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	− (−)	− (−)	− (−)	− (−)	+ (−)
AS9AP1	HF9AP	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (−)	− (+)	+ (+)	− (−)	− (−)
AS9AP2	HF9AP	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (−)	− (+)	+ (+)	− (−)	− (−)
'Spunta'		+	+	−	−	−	−	−	−	−	−	−

[a]) t_R [min].

Sanford and *Sinden* [21] suggested that GA production is polygenic, with high broad sense heritability. In the cultivated potato, it has been hypothesized that GA expression is partially modulated by two interacting loci [22]. Molecular studies by *Yencho et al.* [23] allowed the identification of several QTLs in segregating *S. tuberosum–S. berthaultii* hybrids.

Soluble proteins and hydrophilic antioxidant activities (expressed as μg/ml of ascorbic acid) of *S. bulbocastanum–S. tuberosum* haploids varied greatly (*Table 3*). The former ranged from 1.5 mg/100 g (AS9AP2) to 9.3 mg/100 g (AS6A5), the latter from 6.0 μg/ml (AS6A1) to 10.8 μg/ml (AS6A6). It is worth noting that, except for AS9AP2, both soluble proteins and hydrophilic antioxidant activities were always higher in haploids compared to their parents. This suggests that, in terms of soluble proteins and ascorbic acid pathways, the 24-chromosome diploid condition of our haploids is more advantageous than the 48-chromosome tetraploid condition of their parents. As known, the merging of divergent genomes and/or increasing ploidy levels cause a number of genetic changes that influence gene expression and function as well as genome organization [24]. These changes can be more dramatic at the tetraploid level. As reported for other traits [25], it is also possible that a heterotic threshold exists for these two traits. If this is true, then haploidization may have created the optimal level of heterozygosity, not necessarily equivalent to the maximum level. Work is in progress to verify whether what we found for soluble protein and hydrophilic antioxidant activity holds true also for GA. Therefore, we are currently quantifying the GA content of our haploids and that of their parents. In *S. tuberosum–S. acaule* somatic hybrids, a positive correlation was found between GA and ploidy level [26].

Three main factors were revealed by this research. First, following meiosis, the GA pattern of potato haploids drastically changed compared to that of parents. This has implications relevant to applied breeding when biodiversity is used. Second, new GAs

Table 3. *Soluble Proteins* (mg/100 g tuber fresh weight) *and Hydrophilic Antioxidant Activity* (expressed as µg/ml of ascorbic acid) *in Tubers of Haploids Extracted from* Solanum bulbocastanum (+) S. tuberosum *Somatic Hybrids and of* S. tuberosum *Control Spunta*

Haploid	Parental somatic hybrid	Soluble proteins [mg/100 g fresh tuber]	Antioxidant activity [µg/g dried tuber]
AS5A1	HF5A	3.2 (147)[a]	7.8 (179)
AS6A1	HF6A	5.7 (46)	6.0 (362)
AS6A2	HF6A	6.0 (54)	8.9 (585)
AS6A4	HF6A	5.9 (51)	10.0 (669)
AS6A5	HF6A	9.3 (139)	10.0 (669)
AS6A6	HF6A	7.8 (100)	10.8 (731)
AS6E1	HF6E	4.4 (29)	10.7 (494)
AS9I2	HF9I	1.8 (5.9)	10.6 (108)
AS9AP1	HF9AP	1.9 (19)	8.5 (118)
AS9AP2	HF9AP	1.5 (– 6.3)	7.6 (95)
'Spunta'		3.8	13.9

[a]) In parenthesis, the variation [%] with respect to the parental somatic hybrid.

were found in haploids, possibly due to combinatorial biochemistry events. They may represent compounds with potential pharmaceutical interest. Third, for some metabolites, it is possible that the 24-chromosome condition of our haploids can be genetically preferable to the tetraploid one.

Experimental Part

General. UV Spectra: *Perkin-Elmer Lambda 25* UV/VIS spectrophotometer. Anal. TLC: silica gel (*Kieselgel 60 F_{254}*; 0.25 *Merck*); spots were visualized by exposure to I_2 vapors or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH or with H_2CrO_4, followed by heating at 110° for 10 min.

Plant Materials. Ten *S. bulbocastanum* (+) *S. tuberosum* haploids, their parents, and *S. tuberosum* cv. Spunta were used in this study. Haploids were regenerated from anther culture of somatic hybrids HF5A (haploid AS5A1), HF6A (haploids AS6A1, AS6A2, AS6A4, AS6A5, AS6A6), HF6E (haploid AS6E1), HF9I (haploid AS9I2), and HF9AP (haploids AS9AP1, AS9AP2). Three plants per genotype were grown in a greenhouse (in 25-cm-diameter pots) under controlled conditions. Five tubers (17–22 g) from each genotype were randomly selected, washed, cut into small cubes *ca.* 1 cm wide (including skin and cortex), and frozen with liquid N_2. The samples were freeze-dried and finely ground. Tubers collected had similar size (*ca.* 5 cm long). The resulting powders were used for analyses.

Glycoalkaloid (GA) *Extraction.* One gram of freeze-dried potato sample was extracted with 2% aq. AcOH. The supernatant was filtered through *Whatman No. 4* and re-extracted under the same conditions. The supernatants for each sample were combined. The soln. was alkalinized with $NH_3 \cdot H_2O$ (30%) and extracted with H_2O/sat. BuOH. The combined extracts were evaporated under reduced pressure by the azeotrope formed by addition of *Milli-Q* H_2O as reported in [11]. The residue was detected by TLC (SiO_2; AcOEt/MeOH/1% NH_3 5:3:2) and re-dissolved in 1 ml of MeOH for direct analysis by HPLC.

HPLC Analysis of GAs. The HPLC instrument (*Shimadzu*, Kyoto, Yapan) consisted of a *Series LC-10AdVP* pump, *FCV-10AlVP* valves, *SPD-10AVVP* spectrophotometric detector, and *DGU-14A* degasser. The HPLC separations were performed using a *Macherey-Nagel* (D-Düren) high-density reversed-phase (RP) *Nucleosil 100–5 C_{18} HD* column (250 × 4.6 mm i.d.; 5 mm) provided with an in-line guard column from *Alltech* (I-Sedriano). Anal. and HPLC-grade solvents for chromatographic use were

purchased from *Carlo Erba* (I-Milan). H_2O was of HPLC-grade, obtained by a *Milli-Q* system *Millipore* (Bedford, MA, USA). Disposable syringe filters, *Anotop* 10–0.2 mm, were purchased from *Whatman* (Maidstone, UK). The tuber alkaline org. extracts, obtained as described above, were dissolved in MeOH to have a concentration of 20 mg/ml. Ten ml of these solns. were injected by a 20-ml loop. GAs were eluted with MeCN/0.1% CF_3COOH 3:7 at a flow rate of 1 ml/min. The UV detector (*Shimadzu*) was set at 208 nm.

Antioxidant Activity. The DMPD (= *N*,*N*-dimethyl-*p*-phenylenediamine) method [27] was used to determine the H_2O-soluble antioxidant activity. Two hundred mg of freeze dried potato samples were extracted twice with 5 ml of H_2O. The supernatants, obtained after centrifugation at 4000 rpm for 10 min, were combined and filtered through *Whatman No. 4* filter paper. Two ml of this soln. were added to the soln. containing the DMPD radical cation in acetate buffer. The absorbance quenching at 505 nm was compared with that obtained by a standard soln. of ascorbic acid. The ABTS ((2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) method, performed as described by *Pellegrini et al.* [28], was employed to assess the lipophilic antioxidant activity. The solid residue of H_2O extraction was extracted with 5 ml of acetone. The slurry was centrifuged at 4000 rpm for 10 min. The residues were re-extracted under the same conditions, and the supernatants were combined. Five hundred µl of this extract were added to a soln. of ABTS cation. The absorbance quenching at 734 nm was compared with that obtained by a standard soln. of *Trolox* [28].

Total Soluble Proteins Analysis. The same H_2O extracts used for antioxidant-activity analysis were employed for total soluble protein determination. Proteins were determined using 10 µl of this soln. according to *Bradford* [29] using BSA as standard.

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