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Impact of Ploidy Change on Secondary Metabolites and Photochemical Efficiency in *Solanum bulbocastanum*

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Plants are well known for producing a wide diversity of natural compounds and several strategies have been proposed to enhance their production. Among them, somatic chromosome doubling may represent an effective and inexpensive method. The objective of the current study was to investigate the effect of polyploidization on the leaf metabolic profile and content of tetraploids produced from a wild diploid (2n=2x=24) potato species, *Solanum bulbocastanum* Dun. Photochemical efficiency of tetraploids was also analyzed. Results from HPLC-DAD and LC/MS analyses provided evidence that tetraploid genotypes displayed either a similar or a lower phenylpropanoids, tryptophan, tyrosine and α-chaconine content compared with the diploid parent. Similarly, no significant differences were found among genotypes both for measures of gas and for chlorophyll fluorescence, except for non-photochemical quenching (NPQ). Steroidal saponins content revealed superiority of some tetraploids with respect to the diploid parent, suggesting perturbations in the mechanism regulating the biosynthesis of such compounds following polyploidization. Lack of superiority may be attributed to the time required for adjustment, adaptation and evolution after the genomic shock induced by polyploidization, as well as the fact that an optimum ploidy level for each species may be crucial. Our results suggest that polyploidization as a strategy to enhance metabolite production cannot be generalized.

Keywords: Glycoalkaloids, Phenylpropanoids, Potato biodiversity, Polyploidy, Gas exchanges.

Plants produce a wide array of secondary metabolites whose role, chemical characteristics and utility for human health, as well as for plant breeding efforts, are receiving attention within the scientific community. Many of these natural compounds are currently used as drugs to treat infections or fight diseases such as cancer, stroke and heart problems. Others, thanks to their adaptive role, can be employed to develop genetically improved crops that resist/tolerate pest and pathogen attacks. Mazid *et al.* [1] suggested that the search for new phytochemicals should be a priority towards a sustainable conservation and use of plant biodiversity. The increased knowledge of plant secondary metabolites, coupled with the low product yield often recorded, has stimulated research on the possibility to increase the level of phytochemicals in plants. Nowadays several options are available, especially in light of the new advances in the field of plant biotechnologies. Through *in vitro* culture of selected explants, secondary metabolites can be produced all year around with several advantages in terms of predictability, purity and political issues (reviewed in [2]). Cell and tissue cultures also allow the use of elicitors that induce or enhance metabolite production. In *Catharanthus roseus*, for example, ajmalicine content was greatly increased through elicitors produced by fungi such as *Aspergillus* and *Fusarium* [3]. Similarly, metabolic engineering can enhance metabolite production through the modification of the pathways of interest. It is essentially based on the overexpression of significant genes or gene silencing. Through an antisense-approach that silenced the first dedicated step in the beta-epsilon branch of carotenoid biosynthesis, Diretto and co-workers [4] greatly increased beta carotene content of potato tubers. In the last few years an additional option based on scaling up the ploidy level of metabolite-producing plants has attracted the interest of scientists [5]. Polyploidy (the phenomenon where species possess more than two complete sets of chromosomes in their somatic cells) is common in flowering plants, with several crops like alfalfa, potato, wheat, cassava, banana, apple and sugar beet being polyploids [6a,b]. Polyploidy often causes the appearance of novel characteristics that are not present in the diploid progenitors or exceed the range of the contributing species [7a-c]. It can influence plant metabolism both in a qualitative and quantitative manner. Tetraploids of *Scutellaria baicalensis*, *Artemisia annua* and *Salvia miltiorrhiza* produced more terpenoids or triterpene-type compounds per gram of tissue than their diploid counterparts [8a, b, c]. Mishra *et al.* [9] reported the use of polyploidy in *Papaver somniferum* as an effective tool for producing plants with high morphine content. However, some other authors found the opposite to be true. Dhawan and Lavania [10], for example, evidenced a lower concentration per unit dry weight of essential oils in tetraploids of *Mentha spicata* with respect to the related diploid. Due to these contrasting results, further studies with artificially induced polyploids may be important to increase our knowledge on the effects of gene dosage on secondary metabolites. The cultivated potato *Solanum tuberosum* is well appreciated for its nutritional value and level of antioxidant compounds such as carotenoids, vitamin C, and phenolics [11]. However, only a few studies are available on tuber-bearing *Solanum* species. They may represent an underestimated source of useful compounds and, therefore, can also be used to transfer the relevant genes into the cultivated gene pool [12].

In the frame of a research project on potato germplasm exploitation through polyploidy induction, we have doubled the chromosome number of *S. bulbocastanum*, a diploid (2n=2x=24) potato species widely used in breeding programs due to its resistance traits. We

have produced tetraploid (2n=4x=48) genotypes and studied the effect of chromosome doubling on the content of selected metabolites and on photochemical efficiency. Since there is only scanty information on the effect of polyploidization in *Solanum*, this research may have implications not only to investigate the events underlying polyploid formation and to study the plasticity of plant genomes, but also for practical breeding goals.

We performed HPLC-DAD and LC-MS analyses to investigate the biochemical behavior of *S. bulbocastanum* synthetic polyploids. Leaf extracts did not present qualitative differences in the metabolite profiles of diploid vs. tetraploid genotypes. Conversely, quantitative analysis of leaf secondary metabolites of diploid progenitor blb1c and its tetraploid derivatives showed significant differences among genotypes (Table 1). In terms of metabolites such as phenylpropanoids, quercitin, tyrosine and trypthophan, tetraploids displayed either a similar or a lower content compared with diploid blb1c. A tetraploid showing a higher metabolite content was never found. This contrasts with studies reported elsewhere. In *Chamomilla recutita* [13], a higher flavonoid content was observed as a consequence of polyploidization. In tetraploid plants of *Atropa belladonna*, Evan [14] obtained an increased production of tropane alkaloids. In tetraploids of *Cinchona succiruba* the same authors found an increase in quinine with respect to diploids. More recently, a similar superiority of tetraploids was found in *Catharanthus roseus* [15] for alkaloids and in *Cymbopogon* spp. [16] for essential oil content. Our results on secondary metabolite analysis in synthetic tetraploids of another potato species, *S. commersonii*, also showed that tetraploids generally had an increased amount of phenylpropanoids compared with the diploid progenitor [17]. To explain the different metabolic consequences of polyploidization in *S. bulbocastanum* and *S. commersonii* it may be hypothesized that in these two species polyploidization differently affected the activity of key enzymes of the biosynthetic pathway of phenylpropanoids. Such a key enzyme can be phenylalanine ammonia lyase (PAL), which catalyzes the first step of the phenylpropanoid pathway and controls the flux into the phenylpropanoid biosynthesis [18]. Further studies should be carried out to test this hypothesis. However, species–dependent variation in esterase activity after genome doubling was observed in several species (*Oryza*, *Citrullus*, *Brassica*, *Aegilops* and *Triticum*) with contrasting evidence [19]. To explain the lack of phenotypic superiority of tetraploids over diploid blb1c it may be hypothesized that plants with higher ploidy may experience fitness costs due to elevated DNA content and replication, defective mitosis, and increased cell size [20]. Since we previously found that our tetraploids underwent epigenetic changes [21], it is possible that these caused modifications in the expression of relevant genes.

In this research we also focused on glycoalkaloid content of tetraploids vs the diploid parent. Since few glycoalkaloid standards are commercially available and most of those are aglycones, we determined the structure of the glycoalkaloids by LC-ESI-MS, according to both the *m/z* values of protonated molecular ions $[M+H]^+$ and the fragment ions detected in the MS/MS experiments. Our results were then compared with those reported in previous studies [22a, b, c]. The majority of glycoalkaloids were present in traces and their amount was not measurable. The only glycoalkaloid that it was possible to quantify was *α*-chaconine, a member of the solanidane family. *α*-Chaconine was eluted at 10 min and showed an $[M+H]^+$ ion at *m/z* 852.0, indicating a molecular formula of $C_{45}H_{73}NO_{14}$ (theoretical *m/z* value 852.06 g mol^{-1}). Fragmentation of this ion produced product ions at *m/z* 706.4 (attributed to a loss of the L-rhamnose monosaccharide), and at *m/z* 560.4 (produced by a loss of the L-rhamnose moiety from the *m/z* 706.4 ion). The next stage in the toxin fragmentation pathways consists of the loss of the last monosaccharide from the ion, *m/z* 560.4, to produce the aglycon ion, *m/z* 398.3, with the water-loss ion, *m/z* 380.3. Our results provided evidence that polyploidization reduced *α*-chaconine content in *S. bulbocastanum*. Compared with diploid blb1c (4.95 mg/100 g), tetraploid genotypes decreased from 67% (blb25) to 36% (blb26) (Table 1). Similar results were found for *S. chacoense*, where the leaf concentrations of total glycoalkaloids were significantly lower in the tetraploids derived from tissue culture of leaf explants [23]. Overall, such reduction of glycoalkaloids may be considered undesired in light of their role in clinical and pharmaceutical applications. Indeed, studies carried out during the past 10 years suggest that naturally occurring glycoalkaloids possess beneficial effects in treating human cancers [24].

Our quali-quantitative study showed the presence of steroidal saponins. Saponins are interesting metabolites for pharmaceutical applications. Indeed, some plant steroids resemble human steroids in both form and function, In particular, some plant saponins can weakly mimic the human hormones that they resemble. Tran *et al.* [25] isolated new spirostanol and furostanol saponins from roots and rhizomes of *Dracaena angustifolia* and reported that these compounds have a potent antiproliferative activity against some tumor cells comparable with that of commercial medicine. Consequently, in the last years, many studies are focusing on new saponin isolation and characterization. Four steroid saponins were detected and quantified in the extracts: β-D-Glucopyranoside, (3β,25R)-26-(β-D-glucopyranosyloxy)furosta-5,20(22)-dien-3-yl-*O*-6-deoxy-α-L-mannopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)] (named GluSter1), β-D-Glucopyranoside,(3β,25R)-26-(β-D-glucopyranosyloxy)furosta-5,20(22)-dien-3-yl-*O*-6-deoxy-α-L-mannopyranosyl-(1→2)-*O*-[6-deoxy-α-L-mannopyranosyl-(1→4)] (named GluSter2), β-D-Glucopyranoside, (3β,25R)-26-(β-D-glucopyranosyloxy)furosta-5,20(22)-dien-3-yl-2-*O*-(6-deoxy-α-L-mannopyranosyl) (named GluSter3), and β-D-Glucopyranoside, (3β,25R)-spirost-5-en-3-yl-*O*-β-D-glucopyranosyl (named GluSter4) (Table 2). These compounds were identified by LC-MS/MS analyses on the basis of their high resolution molecular weight determination and the main fragment ions observed in the MS/MS experiments. In particular, the fragmentation patterns and presence of diagnostic fragment ion at *m/z* 271.2 and 253.2 (detected for all these compounds), indicated that they were spirostanol or furostanol saponins [26]. Moreover, fragment ion at *m/z* 415.3 suggested compounds GluSter1, GluSter2 and GluSter3 as furostanol saponins, whereas the fragment ion at *m/z* 433.3 observed in the MS/MS spectrum of GluSter4 allowed us to identify this compound as a spirostanol saponin [26]. In general, the steroid saponin content of tetraploid blb25 and blb26 was always higher than that observed in diploid blb1c. By contrast, the other two tetraploid genotypes (blb10 and blb22) showed steroid saponin content significantly lower than blb1c. These results on saponin analysis suggested perturbations in the mechanism regulating the biosynthesis of such compounds following polyploidization. It is interesting to underline the outlier role of blb26. It showed the highest amount of steroidal saponins but, on other hand, it displayed the lowest content of phenylpropanoids compared both with the diploid progenitor and to the other tetraploids. These results on blb26 are consistent with previous epigenetic and anatomical data [21], indicating that for some parameters (e.g. hypomethylation frequency of internal DNA cytosine, leaf lamina area, vessel lumen area) this genotype sometimes behaves better than other *S. bulbocastanum* synthetic tetraploids, but sometimes does not. Recently, [27] it has been reported that there are one or more genetic factors capable of sensing the alteration of chromosome number and inducing gene expression alterations. Thus, for each

trait and genotype, the consequences of polyploidization can be different and stochastic. This should be kept in mind, not only in basic studies, but also for practical purposes (e.g. plant breeding). Indeed, it means that genetic variability can be produced following polyploidization, and useful variants can be found. A larger sample of synthetic tetraploids of *S. bulbocastanum* will confirm this hypothesis.

Since polyploidy can influence the photochemical efficiency of a plant, it was of interest to investigate gas exchanges, fluorescence parameters and photochemical pigment production.

No significant differences were found among genotypes for measures of gas exchanges [net CO_2 assimilation (A_{CO2}), stomata conductance (gs), transpiration rate (T)] and chlorophyll fluorescence (maximum quantum yield -Fv/Fm-, effective photochemical efficiency of PSII –ΦPSII- and Electron Transport Rate –ETR-) (Table 3). Only non-photochemical quenching (NPQ) significantly varied. Similarly, spectroscopic analysis of pigment content in leaves of diploid progenitor and its tetraploids did not show any significant difference (Table 3).

It is difficult to generalize the effects of polyploidy on photosynthesis [28]. In spite of the fact that all enzymes involved in the photosynthetic process are present in chloroplasts and polyploid plants have a large number of chloroplasts in the mesophyll cells, several authors reported, as in our case, similar photosynthetic rates between diploid and polyploid plants [29a, b, c]. Also, in terms of pigment production, it was not possible to frame a general rule for the behavior of our synthetic polyploids. In *Vicia villosa* Tulay, Unal [30] observed more chlorophyll in tetraploids. Similar results were reported for black wattle [31], watermelon [32] and garden pansy [33]. By contrast, in *Atriplex confertifolia* chlorophyll content did not change at various levels of ploidy [34].

The findings of our study showed that for several analyzed chemical and physiological parameters, synthetic tetraploids of *S. bulbocastanum* did not show superiority compared with the diploid progenitor. The only exception was steroid saponin content in some tetraploids. Probably in *S. bulbocastanum* most genes exhibit dosage compensation following polyploidization. Lack of consistent tetraploid superiority may also be attributed to the time required for adjustment and adaptation after the genomic shock. Our previous molecular studies provided evidence that alterations in methylation level occurred between diploid and tetraploid genotypes [21], whereas no polymorphism was observed at the genetic level. These aspects can indirectly help to explain the effects of chromosome doubling. It is possible that in *S. bulbocastanum* different homeostatic mechanisms driven by epigenetic modifications occurred to preserve the stability of genomic structure and, probably, to control gene expression and hence metabolic and physiological traits. The stochastic trend observed can be due to significant genotype specific differences in the expression of relevant genes. In *A. thaliana*, Yu *et al*. [27] have already found that response to polyploidization has a genetic basis and has a link with DNA methylation. We are currently undertaking transcriptomic studies to better interpret our phenotypic data. However, in light of the results reported here, and elsewhere, we can hypothesize that polyploidization as a strategy to enhance metabolite production cannot be generalized and should be carefully verified. Indeed, chromosome doubling might generate unpredictable and uncontrollable changes that reduce performances.

Experimental

Plant material: A clone (named blb1c) belonging to accession PI 275190 of diploid (2n=2x=24) *S. bulbocastanum* was used in this study. Included were also four tetraploid (2n=4x=48) clones of blb1c (blb10, blb22, blb25 and blb26) obtained through *in vitro* oryzalin (3,5-dinitro-N^4,N-dipropylsulfate) treatment of non-flowering shoot tips, as previously described [17]. All plants were

Table 1: Secondary metabolite content of *Solanum bulbocastanum* leaves in diploid progenitor (blb1c) and its tetraploid derivatives.

Metabolite	blb1c	blb10	blb22	blb25	blb26
Phenylpropanoids[a]					
Neochlorogenic acid	5.2 ± 0.8 a [b]	2.8 ± 0.7 b	5.3 ± 0.8 a	5.5 ± 0.6 a	0.2 ± 0.2 c
Chlorogenic acid	40.1 ± 13.3 a	27.4 ± 3.1 ab	39.1 ± 2.5 a	24.1 ± 5.2 b	5.8 ± 0.7 c
Caffeic acid	2.4 ± 0.3 ab	2.1 ± 0.2 ab	2.2 ± 0.3 ab	2.6± 0.4 a	1.8 ± 0.3 b
Caffeoylquinic acid	6.5 ± 2.0 a	4.05 ± 0.4 ab	5.7 ± 0.7 a	4.6 ± 0.4 ab	2.4 ± 0.3 b
Me- caffeoyl quinone	8.4 ± 3.7 a	4.1 ± 0.2 bc	6.6 ± 0.8 ab	4.8 ± 1.4 bc	2.2 ± 0.3 c
Caffeoyl putrescine	2.5 ± 0.4 a	2.1 ± 0.08 ab	2.2 ± 0.07 ab	2.6 ± 0.2 a	1.9 ± 0.3 b
Miscellaneous					
Quercitin	1.0 ± 0.4 a	0.6 ± 0.06 bc	0.4 ± 0.1 c	0.9 ± 0.2 ab	N.D.
Tyrosine	3.3 ± 2.0 a	1.4 ± 0.07 b	0.3 ± 0.1 b	3.3 ± 1.3 a	0.04 ± 0.02 b
Tryptophan	4.9 ± 2.2 ab	1.3 ± 0.1 bc	5.6 ± 2.1 a	4.1 ± 1.4 ab	0.3 ± 0.2 c
Glycoalkaloid					
Chaconine	4.9 ± 2.3 a	1.9 ± 0.3 ab	1.7 ± 1.4 b	1.6 ± 0.9 b	3.2± 0.5 ab
Steroid saponins					
GluSter1[c]	48.1±1.2 b	38.3±2.2 c	27.2±1.3 d	34.9±1.9 c	66.5±3.7 a
GluSter2	19.8±0.9 c	15.1±0.8 d	13.7±0.9 d	50.9±3.6 b	72.0±3.9 a
GluSter3	12.2±1.1 c	10.0±0.7 d	6.2±0.7 e	18.2±0.9 b	23.4±1.8 a
GluSter4	11.3±0.7 c	9.0±0.5 d	7.0±0.9 e	16.9±0.9 b	19.1±0.9 a

[a] The metabolite content was expressed as mg/100 g of dried tissue and represented the average number of triplicate determinations (mean ± standard deviation). [b] In each row, means denoted by the same letter did not significantly differ at $P \leq 0.05$ according to the Duncan's multiple range test. [c] GluSter1: β-D-Glucopyranoside, (3β,25R)-26-(β-D-glucopyranosyloxy)furosta-5,20(22)-dien-3-yl-*O*-6-deoxy-α-L-mannopyranosyl-(1→2)-*O*-[β -D-glucopyranosyl -(1→4)]. GluSter2: β -D-Glucopyranoside, (3β,25R)-26-(β-D-glucopyranosyloxy)furosta-5,20(22)-dien-3-yl-*O*-6-deoxy-α-L-mannopyranosyl-(1→2)-*O*-[6-deoxy-α-L-mannopyranosyl-(1→4)]. GluSter3: β-D-Glucopyranoside, (3β,25R)-26-(β-D-glucopyranosyloxy)furosta-5,20(22)-dien-3-yl 2-*O*-(6-deoxy-α-L-mannopyranosyl). GluSter4: β-D-Glucopyranoside, (3β,25R)-spirost-5-en-3-yl -*O*-β-D-glucopyranosyl . N.D.= not detected

Table 2: HR-MS and MS/MS data leading to the identification of the four steroid saponins detected in the metabolic analyses of diploid progenitor blb1c and its tetraploid derivatives.

Compound	Positive ion	Experimental *m/z*[a]	Theoretical *m/z*	Main fragment ions
GluSter1	$C_{51}H_{83}O_{22}^+$	1047.5316	1047.5376	885.5; 739.4; 577.4; 415.3; 271.2; 253.2
GluSter2	$C_{51}H_{83}O_{21}^+$	1031.5373	1031.5427	869.5; 723.4; 577.4; 415.3; 271.2; 253.2
GluSter3	$C_{45}H_{73}O_{17}^+$	885.4827	885.4848	723.4; 577.4; 415.3; 271.2; 253.2
GluSter4	$C_{39}H_{63}O_{13}^+$	739.4265	739.4269	577.4; 433.3; 271.2; 253.2

[a] High resolution MS data.

Table 3: Results on physiological trait analysis in diploid blb1c and three tetraploid derivatives**.** Gas exchanges measures of net CO_2 assimilation -A_{CO2}- (mmol $CO_2m^{-2}s^{-1}$), conductance and transpiration (mmol $HO_2m^{-2}s^{-1}$). Chlorophyll fluorescence measures of Electron Transport Rate (ETR, expressed as $\mu molm^2s^{-1}$), effective photochemical efficiency of PSII (ΦPSII) and maximum quantum yield (Fv/Fm). Pigment content Chla, Chlb and Chl a+b, expressed as mg g^{-1} fresh weight, chlorophyll ratio (Chl a/b) and carotenoids of *Solanum bulbocastanum* leaves in diploid progenitor (blb1c) and its tetraploid derivatives (blb10, blb22, blb25).

Physiological trait[1]	blb1c	blb10	blb22	blb25
Gas exchanges				
A_{co2}	13.63 ± 0.24 a[2]	13.03 ± 1.28a	14.72 ± 0.77a	13.32 ± 0.8a
Conductance	0.11 ± 0.03 a	0.10 ± 0.01 a	0.08 ± 0.01 a	0.11 ± 0.03 a
Transpiration	0.99 ± 0.11 a	0.95 ± 0.10 a	0.94 ± 0.05 a	1.02 ± 0.10 a
Chlorophyll fluorescence				
Fv/Fm	0.80 ± 0.01 a	0.80 ± 0.01 a	0.82 ± 0.01 a	0.80 ± 0.02 a
ΦPSII	0.61 ± 0.02 a	0.59 ± 0.03 a	0.56 ± 0.04 a	0.57 ± 0.02 a
ETR	77.03 ± 2.32 a	75.42 ± 1.91 a	70.68 ± 5.76 a	71.87 ± 3.12 a
NQP	0.81 ± 0.05 a	0.78 ± 0.04 a	0.16 ± 0.02 b	1.15 ± 0.10 b
Pigment content				
Chla	1.22 ± 0.08 a	1.17 ± 0.01 a	1.22 ± 0.01 a	1.07 ± 0.2 a
Chlb	0.39 ± 0.07 a	0.38 ± 0.02 a	0.43 ± 0.04 a	0.36 ± 0.06 a
Chla + Chlb	1.61 ± 0.14 a	1.55 ± 0.03 a	1.65 ± 0.04 a	1.43 ± 0.26 a
Chla/Chlb	3.22 ± 0.53 a	3.15 ± 0.18 a	2.85 ± 0.24 a	2.98 ± 0.11 a
Carotenoids	0.24 ± 0.01 a	0.24 ± 0.01 a	0.22 ± 0.02 a	0.23 ± 0.04 a

[1] Each value of all measured parameter represented the average number of triplicate determinations on three plants/genotype, repeated two times (mean ± standard deviation). [2] In each row, means denoted by the same letter did not significantly differ at $P \leq 0.05$ according to the Duncan's multiple range test.

maintained and propagated *in vitro* on MS medium [35], including salts, vitamins, sucrose (30 g /L) and microagar (9 g/L) and adjusted to pH 5.8. The cultures were maintained in a growth chamber at 25°C ± 2 under a 16/18 (light/dark) photoperiod at 125 μEm^{-2} s^{-1} irradiance provided by a cool white fluorescent tube (TL-D 58W/33-640 1SL, Philips, Eindhoven, the Netherlands). All analyses were performed on 3 plants/genotype, with 3 replicates per measurement, repeated 2 times. Fully developed leaves were selected from plants at the same physiological stage. Plant irrigation was performed 3 times a week depending on the evaporative demand of the environment and, in the case of physiological analysis, with the same amount of water.

Chemicals: Analytical grade *n*-hexane and methanol were obtained from Carlo Erba (Milan, Italy). HPLC grade methanol (MeOH) and formic acid were purchased from Romil (Cambridge, UK). HPLC grade water (18 mV) was prepared using a Millipore (Bedford, MA, USA) Milli-Q purification system. Standards were purchased from Extrasynthese (Lyon, France), and Sigma Aldrich (Milan, Italy); α-solanine, and diosgenin glucoside used as HPLC standards to perform LC-MS/MS quantitative analyses, was purchased from Extrasynthese (Lyon, France).

General methods: HPLC analyses were conducted on an Agilent 1200 series system (Agilent Technologies, Palo Alto, CA, USA), equipped with a binary pump delivery system G-1312, degasser G-1322A, G-1315A Photodiode Array Detector, G-1328A autosampler. ESIMS (positive mode) were obtained using a Q-TOF Premier instrument (Waters, Milford, MA), equipped with an electrospray ion source and coupled to a 2690 Alliance HPLC.

Sample preparation: A representative quantity (0.5 g) of fresh leaves was freeze-dried and defatted with *n*-hexane, and then extracted for 48 h with MeOH by exhaustive maceration (3 x 10 mL). Analyses were performed by injecting 20 µL of 1 mg/mL solutions of each methanol residue Triplicate injections were made. To detect the secondary metabolite composition of leaves, sample preparation and HPLC conditions were adjusted to avoid any detectable degradation of these compounds during the performance. To determine glycoalkaloid content, part of the methanolic extract was diluted to 10% MeOH at a concentration of 0.4 mg/mL. An appropriate volume of internal standard (IS) α-solanine was added to give a final concentration of 0.1 µmol/mL and 100 µL aliquot injected into the analytical system. Steroid saponins were quantified using diosgenin glycoside as an external standard. Samples were kept chilled and not exposed to bright light. All standards were prepared as stock solutions at 10 mg/mL in MeOH, while tyrosine and tryptophan as stock solutions in 0.1 N HCl, and α-solanine in 2.5% metaphosphoric acid.

HPLC-DAD analyses: Analyses were carried out using a monolithic Onyx column (50 x 2 mm, C18) at a flow rate of 1 mL/min, and injecting 20 µL of sample. Gradient elution of 0-1 min 100% buffer A (10 mM formic acid, pH 3.5, with NH_4OH), 1-6.7 min 0-30% buffer B (100% MeOH with 5 mM ammonium formate), 6.7-11.7 min 40-70% buffer B, 11.7-14.56 min 70-100% buffer B, 14.56-20 min 100% buffer B. UV detection was at 210, 280 and 254 nm. The external standard method of calibration was used: for each standard, a six-point calibration curve was obtained. Neochlorogenic acid was evaluated as chlorogenic acid equivalent, and dihydrocaffeoyl polyamines as dihydrocaffeic acid equivalents at A_{210}, but with no adjustment made for the polyamine part of the molecule [22, 36].

LC-MS/MS analyses: To achieve high-accuracy MS and MS/MS measurements, LC-MS/MS analyses were performed on a Q-TOF Premier instrument (Waters, Milford, MA) coupled to a 2690 Alliance HPLC. Instrument accuracy was checked by the external calibration of Glu-Fib. Instrument tuning and mass calibration of the spectrometer were performed using α-solanine ($[M+H]^+$= *m/z* 869.07) as standard. MS/MS data processing involved use of MassLynx 4.1. Chromatographic separation was conducted injecting 100 µL (40 µg) of each extract on a monolithic C18 (50x2 mm, flow rate 1 mL/min) column (Onyx, Phenomenex). Gradient elution of 0-1 min 100% buffer A (10 mM formic acid, pH 3.5, with NH_4OH), 1-6.7 min 0-30% buffer B (100% MeOH with 5 mM ammonium formiate), 6.7-11.7 min 40-70% buffer B, 11.7-14.56 min 70-100% buffer B, 14.56-20 min 100% buffer B. Mass analyses were performed in positive mode, using a dependent MS/MS function. The following instrumental parameters were used: source temperature 80°C, desolvation temperature 180°C, cone gas flow rate 50 L/h, desolvation 300 L/h. Capillary voltage was 3 kV, cone voltage 28, extraction cone 5 while fragmentation voltage was 40 V. The MCP detector was at 2250 V, and the energy adjustable collision cell was filled with pure argon gas. The full mass scan ranged between *m/z* 250 and 1500.

Gas exchanges: Net CO_2 assimilation rate (A_{Co2}), stomata conductance, and transpiration rate were measured *in vivo* in attached leaves of blb1c and three tetraploids (blb10, blb22, blb 25)

using a portable InfraRed Gas Analyzer (IRGA; LI-6400-02B; Li-cor Biosciences; Lincoln, NE, USA) with a cuvette enclosing the leaf area of 6 cm^2. External air was scrubbed with CO_2 and mixed with a supply of pure CO_2 to reach a reference concentration of 360 ppm, with a flow rate of 200 mL min^{-1} and 80% external relative humidity. The internal leaf temperature was maintained at 22 ±1°C (±SD). All measurements were performed between 16.00 and 19.00 h in January and February 2010. The experiment was performed at the Universidad de La Frontera, Departamento de Ciencias Químicas, Facultad de Ingeniería, Ciencias y Administración; Temuco, Chile.

Chlorophyll fluorescence and pigment analysis: *In vivo* measurements of chlorophyll fluorescence of PSII of blb1c and 3 tetraploids (blb10, blb22, blb 25) were made with a portable pulse-amplitude modulated fluorimeter (FMSII, Hansatech Instruments Ltd., Norfolk, UK). Comparable or the same leaves as those used for IRGA determinations were selected for fluorescence analyses. The protocol of Alberdi *et al.* [37] was followed. Definitions of fluorescence parameters Fv/Fm and ΦPSII were used as described by Maxwell and Johnson [38]. After fluorescence measurements, the same leaves were detached and immediately placed in liquid nitrogen and kept frozen at -80°C until pigments analyses. Leaves (100 mg fresh mass) were ground to a fine powder with liquid nitrogen in a cold mortar. About 1 mg of $CaCO_3$ was added before grinding. Leaf pigments were extracted with 96% ethanol according to Lichtenthaler and Wellburn [39]. Total chlorophylls and carotenoids were measured by a spectrophotometer (Genesys 5; Spectronic Unicam, Rochester, NY) at 663 (chlorophyll a), 646 (chlorophyll b), and 470 (chlorophyll b) nm, and pigment concentrations were calculated according to Lichtenthaler and Wellburn [39]. This part of the research was conducted at the Universidad de La Frontera, Departamento de Ciencias Químicas, Facultad de Ingeniería, Ciencias y Administración; Temuco, Chile.

Statistical analysis: Statistical analyses were performed using the 2002 Statistical Package for Social Sciences (SPSS) version 11.5 for Windows. Analysis of variance (ANOVA) was used to evaluate differences among all genotypes in metabolite content and physiological performance. Mean separation was performed using the Duncan test with $P \leq 0.05$ probability level.

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References

[1] Mazid M, Khan TA, Mohammad F. (**2011**) Role of secondary metabolites in defense mechanisms of plants. *Biology and Medicine*, Special Issue, ***3***, 232-249.

[2] Karuppusamy S. (**2009**) A review on trends in production of secondary metabolites from higher plants by *in vitro* tissue, organ and cell cultures. *Journal of Medicinal Plants Research*, ***3***, 1222‑1239.

[3] Namdeo A, Patil S, Fulzele DP. (**2002**) Influence of fungal elicitors on production of ajmalicine by cell cultures of *Catharanthus roseus*. *Biotechnology Progress*, ***18***, 159-162.

[4] Diretto G, Tavazza R, Welsch R, Pizzichini D, Mourgues F, Papacchioli V, Beyer P, Giuliano G. (**2006**) Metabolic engineering of potato tuber carotenoids through tuber-specific silencing of lycopene epsilon cyclase. *BMC Plant Biology*, ***6***, 13.

[5] Dhawan OP, Lavania UC. (**1996**) Enhancing the productivity of secondary metabolites via induced polyploidy: a review. *Euphytica*, ***87***, 81-89.

[6] (a) Aversano R, Ercolano MR, Caruso I, Fasano C, Rosellini D, Carputo D. (**2012**) Molecular tools for exploring polyploid genomes in plants. *International Journal of Molecular Sciences*, ***13***, 10316-10335; (b) Flagel LE, Wendel JF. (**2009**) Gene duplication and evolutionary novelty in plants. *New Phytologist*, ***183***, 557-564.

[7] (a) Ainouche ML, Parisod C, Salmon A, Zerjal T, Tenaillon M, Grandbastien MA. (**2009**) Rapid structural and epigenetic reorganization near transposable elements in hybrid and allopolyploid genomes in *Spartina*. *New Phytologist*, ***184***, 1003-1015; (b) Fawcett JA, Maere S, Van de Peer Y. (**2009**) Plants with double genomes might have had a better chance to survive the Cretaceous-Tertiary extinction event. *Proceedings of the National Academy of Sciences USA*, ***106***, 5737-5742; (c) Ramsey J, Schemske DW. (**2002**) Neopolyploidy in flowering plants. *Annual Review of Ecology, Evolution and Systematics*, ***33***, 589-639.

[8] (a) Gao SL, Chen BJ, Zhu DN. (**2002**) *In vitro* production and identification of autotetraploids of *Scutellaria baicalensis*. *Plant Cell Tissue and Organ Culture*, ***70***, 289-293; (b) Banyai W, Sangthong R, Karaket N, Inthima P, Mii M, Supaibulwatana K. (**2010**) Overproduction of artemisinin in tetraploid *Artemisia annua* L. *Plant Biotechnology*, ***27***, 427-433; (c) Gao SL, Zhu DN, Cai ZH, Xu DR. (**1996**) Autotetraploid plants from colchicine treated bud culture of *Salvia miltiorrhiza* Bge. *Plant Cell Tissue and Organ Culture*, ***47***, 73–77

[9] Mishra BK, Pathak S, Sharma A, Trivedi PK, Shukla S. (**2010**) Modulated gene expression in newly synthesized autotetraploid of *Papaver somniferum* L. *South African Journal of Botany*, ***76***, 447-452.

[10] Lavania UC. (**2005**) Genomic and ploidy manipulation for enhanced production of phyto-pharmaceuticals. *Plant Genetic Resources*, ***3***, 170-177.

[11] Camire ME, Kubow S, Donnelly DJ. (2009) Potatoes and human health. *Critical Reviews in Food Science and Nutrition*, ***49***, 823-840.

[12] Hale AL, Reddivari L, Nzaramba MN, Bamberg JB, Miller JC Jr. (**2008**) Interspecific variability for antioxidant activity and phenolic content among *Solanum* species. *American Journal of Potato Research*, ***85***, 332–341.

[13] Svehlíková V, Repcák M. (**2000**) Variation of apigenin quantity in diploid and tetraploid *Chamomilla recutita* (L.) Rauschert. *Plant Biology*, ***2***, 403–407.

[14] Evan WC. (**2009**) Principles related to the commercial production, quality and standardization of natural products (16th ed). In *Trease and Evans' Pharmacognosy*. Evans WC. (Ed). English Language Book Society, Bailliere Tindal, London UK. 81–132.

[15] Xing S-H, Guo X-B, Wang Q, Pan Q-F, Tian Y-S, Liu P, Zhao J-Y, Wang G-F, Sun X-F, Tang K-X. (**2011**) Induction and flow cytometry identification of tetraploids from seed-derived explants through colchicine treatments in *Catharanthus roseus* (L.) G. Don. *Journal of Biomedicine and Biotechnology, **2011***, 1-10.

[16] Lavania UC, Srivastava S, Lavania S, Basu S, Misra NK, Mukai Y. (**2012**) Autopolyploidy differentially influences body size in plants, but facilitates enhanced accumulation of secondary metabolites, causing increased cytosine methylation. *The Plant Journal*, ***71***, 539–549.

[17] Caruso I, Lepore L, De Tommasi N, Dal Piaz F, Aversano R, Garramone R, Carputo D. (**2011**) Secondary metabolite profile in induced tetraploids of wild *Solanum commersonii* Dun. *Chemistry & Biodiversity*, ***8***, 2226-2237.

[18] Howles PA, Sewalt VJH, Paiva NL, Elkind Y, Bate NJ, Lamb C, Dixon RA. (**1996**) Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. *Plant Physiology*, ***112***, 1617-1624.

[19] Nakai Y, Ochiai H, Uchida M. (**1977**) Fine structure of ependymal cells in the median eminence of the frog and mouse revealed by freeze-etching. *Cell and Tissue Research*, ***181***, 311–318.

[20] Stupar RM, Bhaskar PB, Yandell BS, Rensink WA, Hart AL, Ouyang S, Veilleux RE, Busse JS, Erhardt RJ, Buell CR, Jiang J. (**2007**) Phenotypic and transcriptomic changes associated with potato autopolyploidization. *Genetics*, ***176***, 2055-2067.

[21] Aversano R, Caruso I, Aronne G, De Micco V, Scognamiglio N, Carputo D. (**2013**) Stochastic changes affect *Solanum* wild species following autopolyploidization. *Journal of Experimental Botany*, ***64***, 625–635.

[22] (a) Shakya R, Navarre DA. (**2006**) Rapid screening of ascorbic acid, glycoalkaloids, and phenolics in potato using high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*, ***54***, 5253-5260; (b) Shakya R, Navarre DA. (**2008**) LC-MS analysis of solanidane glycoalkaloid diversity among tubers of four wild potato species and three cultivars (*Solanum tuberosum*). *Journal of Agricultural and Food Chemistry*, ***56***, 6949-6958; (c) Vaananen T, Ikonen T, Rokka VM, Kuronen P, Serimaa R, Ollilainen V. (**2005**) Influence of incorporated wild *Solanum* genomes on potato properties in terms of starch nanostructure and glycoalkaloids content. *Journal of Agricultural and Food Chemistry*, ***53***, 5313-5325.

[23] Sanford LL, Kobayashi RS, Deahl KL, Sinden SL. (**1997**) Diploid and tetraploid *Solanum chacoense* genotypes that synthesize leptine glycoalkaloids and deter feeding by Colorado potato beetle. *American Potato Journal*, ***74***, 15-21.

[24] Friedman M. (**2006**) Potato glycoalkaloids and metabolites: roles in the plant and in the diet. *Journal of Agricultural Food Chemistry*, ***54***, 8655−8681.

[25] Tran QL, Tezuka Y, Banskota AH, Tran QK, Saiki I, Kadota S. (**2001**) New spirostanol steroids and steroidal saponins from roots and rhizomes of *Dracaena angustifolia* and their antiproliferative activity. *Journal of Natural Products*, ***64***, 1127–1132.

[26] Li R, Zhou Y, Wu Z, Ding L. (**2006**) ESI-QqTOF-MS/MS and APCI-IT-MS/MS analysis of steroid saponins from the rhizomes of *Dioscorea panthaica. Journal of Mass Spectrometry*, ***41***, 1-22.

[27] Yu Z, Haberer G, Matthes M, Rattei T, Mayer KFX, Gierl A, Torres-Ruiz RA (**2010**) Impact of natural genetic variation on the transcriptome of autotetraploid *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences USA*, ***41***, 17809-17814.

[28] Vyas P, Bisht MS, Miyazawa SI, Yano S, Noguchi K, Terashima I, Funayama-Noguchi S. (**2007**) Effects of polyploidy on photosynthetic properties and anatomy in leaves of *Phlox drummondii*. *Functional Plant Biology*, ***34***, 673–682.

[29] (a) Mauer J, Mayo JM, Denford K. (**1978**) Comparative ecophysiology of the chromosome races in *Viola adunca*. J.E. Smith. *Oecologia*, ***35***, 91–104; (b) Pfeiffer T, Schrader LE, Bingham ET. (**1980**) Physiological comparison of isogenic diploid-tetraploid, tetraploid-octaploid alfalfa populations. *Crop Science*, ***20***, 299–303; (c) Warner DA, Edwards GE. (**1988**) C4 photosynthesis and leaf anatomy in diploid and autotetraploid *Pennisetum americanum* (pearl millet). *Plant Science*, ***56***, 85-92.

[30] Tulay E, Unal M. (**2010**) Production of colchicine induced tetraploids in *Vicia villosa* Roth. *Caryologia*, ***63***, 292-303.

[31] Mathura S, Fossey A, Beck SL. (**2006**) Comparative study of chlorophyll content in diploid and tetraploid black wattle (*Acacia mearnsii*) *Forestry*, ***79***, 381-388.

[32] Jaskani MJ, Kwon SW, Kim DH. (**2005**) Comparative study on vegetative, reproductive and qualitative traits of seven diploid and tetraploid watermelon lines. *Euphytica*, ***145***, 259-268.

[33] Lagibo AD, Kobza F, Suchánková P. (**2005**) Polyploidy effects on frost tolerance and winter survival of garden pansy genotypes. *Horticultural Science* (Prague), ***32***, 138–146.

[34] Warner DA, Edwards GE. (**1989**) Effects of polyploidy on photosynthetic rates, photosynthetic enzymes, contents of DNA, chlorophyll, and sizes and numbers of photosynthetic cells in the C_4 dicot *Atriplex confertifolia*. *Plant Physiology*, ***91***, 1143-1151.

[35] Murashige T, Skoog F. (**1962**) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Plant Physiology*, ***15***, 473-497.

[36] Parr AJ, Mellon FA, Colquhoun IJ, Davies HV. (**2005**) Dihydrocaffeoyl polyamines (kukoamine and allies) in potato (*Solanum tuberosum*) tubers detected during metabolite profiling. *Journal of Agricultural and Food Chemistry*, ***53***, 5461-5466.

[37] Alberdi M, Reyes-Díaz M, Zúñiga R, Hess S, Bravo LA, Corcuera LJ. (**2009**) Photochemical efficiency of PSII and photoprotective pigments in seedlings and adults of two *Proteaceae* with different shade tolerance from the Chilean temperate rain forest. *Revista Chilena De Historia Natural*, ***82***, 387-402.

[38] Maxwell K, Johnson GN. (**2000**) Chlorophyll fluorescence-a practical guide. *Journal of Experimental Botany*, ***51***, 659-668.

[39] Lichtenthaler H, Wellburn AR. (**1983**) Determinations of total carotenoids and chlorophyll a and b of leaf extracts in different solvents. *Biochemical Society Transactions*, ***603***, 591–592.