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Cold Treatment Modulates Changes in Primary Metabolites and Flowering of Cut Flower Tulip Hybrids

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Abstract: Tulip is one of the most important bulbous genera in the world's floriculture. It is known that cold exposure of bulbs before planting is required to break the bulb dormancy and to promote the plant's flowering. Preparation procedures performed by breeders differ in the duration and the thermal level, and the choice of the procedure depends on the genotype's sensitivity to temperature; however, little is known about the metabolic responses underlying the different behaviours of the numerous commercial hybrids. We evaluated the influence of two bulb-preparation procedures, 15–18 weeks at 5 ÷ 9 °C, and 9–14 weeks at 2 ÷ 5 °C, in two hybrids of tulip (*Tulipa gesneriana* L.), 'Royal Virgin' and 'Ad Rem', grown hydroponically in a floating system. Tulip plants of the two hybrids responded differently to bulb exposure to low temperatures in terms of early flowering, as this was unaffected by the preparation procedure in 'Royal Virgin' (27.1 days from transplanting, on average), while it was earlier after treatment at higher temperatures compared with lower temperatures in 'Ad Rem' (24.1 vs. 26.7 days at 5 °C vs. at 9 °C). This different flowering earliness may be related to the diverse metabolic responses enacted by the bulbs for cold acclimation that depended on hybrid x thermal treatment. Plant leaf area and flower stem characteristics were similar in the hybrids and were unaffected by the bulb-preparation procedure.

Keywords: geophytes; cold requirement; bulbs; hydroponics; metabolic profile

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1. Introduction

Tulip (*Tulipa* spp., family Liliaceae) is one of the most important genera among cut flowers and ornamental plants in the world's floriculture [1]. Intensive hybridisation, conducted primarily by growers in the Netherlands over the centuries, has resulted in a multitude of tulip cultivars and hybrids, producing plants suitable for a wide range of cultivation conditions and a huge variety of flower shapes and colours, very appreciated as all-year-round cut flower and as a spring-flowering plant in pots, gardens, and parks.

The tulip bulb has an annual replacement cycle, consisting in three phases starting from the bulb planting, as follows: the root growth, the plant growth, and the flowering. These are accompanied by the aerial part senescence, the mother bulb shrivelling, and the daughter bulb dormancy [2]. In plants in a wild environment under Mediterranean and temperate climates, this cycle starts with the floral meristem initiation and differentiation in summer, the flower bud development in winter, and the anthesis in spring. Periodical dissections of the tulip bulb during dormancy shows that an active organogenesis (differentiation of floral and vegetative buds within the bulb scales and of root primordia in the basal plate) takes place during this rest period [3]. In particular, Mulder and Luyten [3] described seven different distinct stages of flower bud differentiation, from phase I (vegetative apex), through phases II, P1, P2, A1, and A2, to phase G (formation of trilobed gynoecium).

For the production of propagation material, daughter bulbs need to reach a minimum dimension to sustain a proper plant growth and the flowering process when transplanted. In the flower bulb trade, bulb size is given in centimetres of circumference and the largest grade is 12/+, as this size ensures the accumulation of a great amount of reserve material and thus promotes the earlier flowering of larger flowers compared with smaller bulb sizes.

After lifting, bulbs must first be exposed to sufficient warmth for a proper flower initiation, then they need a period of cold before planting, to break dormancy and to promote the stem elongation and flower anthesis [2]. The absence of cold treatment of tulip bulbs results in a slow shoot growth and severe flowering disorders, and the duration and temperature required for the dormancy release and the normal flowering process are genotype-specific [2].

In flowering-sized bulbs, flower initiation starts during daughter bulb enlargement and finishes during bulb storage. After its completion, the rate of flower development is greatly affected by environmental factors.

Starch is the main reserve of tulip bulbs; however, they contain also fructans, as secondary reserve, and simple sugars, such as sucrose, glucose, and fructose [4]. The advantage of accumulating fructans instead of only starch depends on the fructans' higher water solubility [5], and on their ability to release hexose sugars via hydrolyzation, available for taking part, when required, in the stabilization of membranes and osmotic adjustment associated with rehydration [6,7]. Cold exposure, increasing the activity of α-amylase [8], promotes starch breakdown in the scales and glucose allocation in the shoot during its elongation, thus favouring water redistribution between scales and buds of tulips ([9], and references therein). Therefore, the accumulation of dry matter and water bonding in the cells of the central bud are enhanced by low temperatures (precooling), but they are inhibited by high temperatures by means of a still-unknown mechanism [9]. Indeed, hormone metabolism pathways involving the gibberellin (GA) and abscisic acid (ABA) as antagonists [10] play major roles in dormancy-release-modulating nitrogen (synthesis and accumulation of proteins and amino acids) and carbon (breakdown of starch, hydrolysis of fructans, and soluble sugars reallocation) metabolism in tulip bulbs ([4], and references therein).

The tulip assortment includes thousands of varieties and hybrids, classified as early-, mid–early-, and late-flowering types, and several hundreds of these are used for forcing. The international register of the Royal General Dutch Bulbgrowers Society [11] classifies tulips genotypes in 15 groups, based on the plant dimensions and the flower morphology.

In regions where winter temperatures are cold enough, tulip bulbs planted in the field are subjected to the cold period under natural conditions, while bulbs that receive a cold treatment before winter can be forced to flower before their natural time. For instance, cut tulips marketed from September to November, called "ice tulips", are produced from bulbs stored at low temperatures for an extended period.

Cold exposure anticipates the flowering of tulip plants, as in many other spring-flowering ornamental geophytes, and this advance depends on the thermal level of cold treatment and the time of exposure [12]. Cold treatments differ in the time of exposure and the thermal level, depending on the genotype-specific sensitivity of the hybrid and the farm production schedule, and can be schematically ascribed to two approaches, in which the cold requirement is fulfilled in part during the storage and in part after planting, or it is satisfied completely before planting. The most common protocols adopted by breeders are at 9 °C and at 5 °C. The procedure at 9 °C consists of 15–18 weeks at 5 ÷ 9 °C and allows for an earlier flowering, while in 5 °C tulips the procedure requires 9–14 weeks at 2 ÷ 5 °C and allows a slower flowering.

Thanks to a high degree of professional expertise, the latest production technologies, and continuous research and development activities, breeders collect information about the response of each variety to the different forcing methods and are provided with complete information about the best cultivation and environmental conditions for each genotype. However, little is known about the metabolic responses underlying the different behaviours of the numerous commercial hybrids.

We evaluated the influence of the two bulb-preparation procedures, at 9 °C and at 5 °C, in two hybrids of *Tulipa gesneriana* L., belonging to different tulip groups, grown hydroponically in a floating system, on plant growth, flowering earliness, cut flower characteristics, and metabolic profiles during the cultivation cycle, in both the bulbs and the leaves.

2. Materials and Methods

The experiment was carried out in a heated greenhouse equipped with a hydroponic floating system (open loop), at the farm "Tulipani freschi italiani" in Pompei (Naples).

2.1. Plant Material and Culture

Bulbs of the *Tulipa gesneriana* L. hybrids 'Royal Virgin' (H1) and 'Ad Rem' (H2) (size 12/+, P. Aker B.V., The Netherlands) were subjected to two preparation procedures.

Hybrids 'Royal Virgin' (white flower) belongs to the Triumph group, while 'Ad Rem' (with nuanced orange-yellow flower) is a Darwin hybrid (Royal General Dutch Bulbgrowers Society classification) [11]. The 5 °C procedure lasted 12 weeks starting on 12 October (temperature 2 ÷ 5 °C) in both the hybrids, while the 9 °C procedure lasted 17 weeks in 'Ad Rem', from September 7, and 14 weeks in 'Royal Virgin' from 28 September (temperature 5 ÷ 9 °C), according to the different cold requirements of the hybrids. Cold treatments were carried out at the breeding laboratory, before sale, and they were followed by 16 days at 5 °C in refrigerating rooting room, then floating trays were moved to the greenhouse on 4 January. Confirmed pH were measured with a portable pH-EC multiparameter sensor (9811-5 series, Hanna Instruments Intl., Woonsocket, RI, USA).

2.2. Plant Growth and Flowering

Plant growth was monitored weekly on 10 plants per combination *Hybrid* × *Preparation procedure* (40 plants in total), by measuring the flower stem height and the number of leaves. Plant leaf area was measured in fully developed plants at the harvest, with an electronic area meter (Delta-T Devices Ltd., Cambridge, UK).

The flowering process was studied in terms of beginning of flowering (days from transplanting for commercial maturity of flower stems) and duration of flowering (100% of flowering plants), on 39 plants per combination *Hybrid* × *Preparation procedure*.

At the harvest, in 10 flowers per treatment, stem length was measured with a ruler, and stem diameter was calculated as an average of two cross measurements with an electronic calliper. Fresh weight and dry weight (after oven-drying for 48 h at 70 °C) of the cut flower stem and dry matter partitioning in the plant organs (leaves, stem, flower bud, and bulb) were measured with an electronic balance.

2.3. Macronutrients Content in Leaves

The main ion concentration (N, P, K) in leaves and bulbs was determined at the harvest, on the water extract of dry matter [13], using a spectrophotometer (Hach, 2000, Hach Company, Loveland, CO, USA). Organic nitrogen was determined by the Kjeldahl method [14]. Nutrient analyses were carried out on 3 replicates per combination *Hybrid* × *Preparation procedure*.

2.4. Metabolic Profile

2.4.1. Starch and Soluble Carbohydrate Analysis

Starch and soluble sugars were extracted according to Dell'Aversana et al. [15] with some modifications. Frozen powdered tuberous roots and leaves (10 mg) were submitted to two subsequent extractions with 150 mL ethanol 80% (v:v) and a final extraction with 50 mL ethanol 50% (v:v) at 80 °C for 20 min. The tubes were cooled in ice and centrifuged at 14,000× *g* for 10 min at 4 °C. The clear supernatants of the three following extractions were pooled together and stored at −20 °C until analysis. The starch present in the pellets of the ethanol extracts was hydrolysed by addition of 250 mL of 0.1 M KOH and

heating at 95 °C for 2 h. After cooling on ice, the samples were acidified to pH 4.5, mixed 1:1 with a hydrolysis buffer containing sodium acetate 50 mM pH 4.8, a-amylase 2 U/mL, and amyloglucosidase 20 U/mL, and incubated at 37 °C for 18 h. The samples were centrifuged at 13,000× *g* for 10 min at 4 °C, and the supernatant containing the glucose, derived from hydrolysed starch, was used for measurement. The content of glucose, fructose, and sucrose in the ethanolic extracts, and the glucose derived by starch, were determined by an enzymatic assay coupled with reduction in pyridine nucleotides according to Carillo et al. [16]. The content of sugars was expressed as μmol g^{-1} DW.

2.4.2. Soluble Proteins: Free Amino Acid Analysis

Lyophilized samples (10 mg) were extracted with a buffer containing 200 mM TRIS-HCl pH 7.5 and 500 mM $MgCl_2$ at 4 °C for 24 h. The clear supernatants (10 μL) were added to 190 μL of protein assay dye reagent concentrate (Bio-Rad, Milan, Italy) diluted with H_2O milli Q (1:5 v:v) [17]. The soluble protein content in the samples was calculated by comparison with standard curves obtained using known concentrations of bovine serum albumin (BSA) as the reference standard. Proteins were expressed as mg g^{-1} DW.

Free amino acids (μmol g^{-1} DW) were extracted from 10 mg of lyophilized samples in 1 mL ethanol:water (40:60 v:v) overnight at 4 °C, and estimated by HPLC after pre-column derivatization with *o*-phthaldialdehyde (OPA) according to Dell'Aversana et al. [15]. Proline was determined in the same ethanolic extract according to Carillo et al. [16].

2.4.3. Polyphenols Analysis

The total phenol content was determined according to Singleton et al. [18] with some modifications [19]. Lyophilized samples (30 mg) were suspended in 700 μL of 60% methanol (v:v), then centrifuged at 25 °C for 10 min at 13,000× *g*. Aliquots of the clear supernatant (35 μL) were added to 125 μL of the Folin–Ciocalteu reagent diluted with H_2O milli Q (1:1 v:v) and 650 μL of 3% (w:v) sodium carbonate. After 90 min of reaction at room temperature, the absorbance at 760 nm was determined by a Synergy HT spectrophotometer (BioTEK Instruments, Bad Friedrichshall, Germany). The total phenol content in the samples was evaluated with a standard curve obtained using known concentrations of gallic acid (GAE) as a standard. Total phenols were expressed as mg GAE g^{-1} DW.

2.4.4. Chlorophylls and Carotenoids Analysis

A measure of 10 mg lyophilized leaf tissues was extracted in 1 mL methanol according to Wellburn [20] and centrifuged at 13,500 rpm for 10′. Chlorophyll a, chlorophyll b, and total carotenoids were estimated by measuring the absorbance of the supernatants at 665, 652, and 470 nm, respectively, by a microplate reader (Synergy HT, BioTEK Instruments, Bad Friedrichshall, Germany), and expressed as μg g^{-1} DW.

2.5. Statistical Analysis

The experiment was conducted on 39 plants per combination *Hybrid* × *Preparation procedure*, distributed in 3 groups of 13 plants, in randomized blocks. Data were analysed by a two-way ANOVA using SPSS 25 software package (www.ibm.com/software/analytics/spss, accessed on 10 January 2022) and means were compared by Duncan post hoc test at $p \leq 0.05$ and $p \leq 0.01$. The following number of samples were used for each parameter: 3 plants for plant growth parameters, 39 plants for flowering earliness and duration, 10 samples for flower stem characteristics, and 3 plants for metabolites content in leaves and bulbs.

The heat map was generated using the https://biit.cs.ut.ee/clustvis/ (accessed on 12 March 2022) online program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage.

A principal component analysis (PCA) was applied to all the analysed parameters using Minitab® 18.1 statistical software to investigate how the dominant parameters clustered according to the hybrids and the preparation procedures [21]. The score plot and loading matrix were determined based on the first and second principal components (PCs) [16].

3. Results

3.1. Plant Growth and Flowering

Tulip plants of 'Royal Virgin' (H1) and 'Ad Rem' (H2), grown hydroponically, responded differently to preparation procedures of bulbs in terms of flowering earliness, as the time for flowering was unaffected by the preparation temperature in 'Royal Virgin' (27.3 ± 0.16 at 5 °C vs. 26.9 ± 0.16 at 9 °C), while it was 2.6 days shorter after treatment at higher temperature in 'Ad Rem' (24.1 ± 0.14 at 9 °C vs. 26.7 ± 0.15 at 5 °C).

Plant growth in terms of total leaf area and flower stem characteristics were similar in the hybrids and were unaffected by the bulb-preparation procedures (Table 1). Indeed, plants of 'Royal Virgin' seemed to reach a smaller leaf surface (−20%) when bulbs were exposed to cooler temperature (5 °C compared with 9 °C); however, this difference was found to be insignificant (Table 1). Similarly, in this hybrid flower, stem dimensions and fresh weight at the harvest did not differ after the two cold treatments (Table 1). Conversely, in the hybrid 'Ad Rem', the procedure at 5 °C seemed to increase the plant leaf area compared with that at 9 °C; however, in this case also, the difference was found to be insignificant. In this hybrid, cooler temperature significantly reduced the flower stem height (Table 1).

Table 1. Plant growth parameters, fresh and dry weight, and dry matter partitioning in the different organs in plants of the hybrids of *Tulipa gesneriana* L. 'Royal Virgin' and 'Ad Rem', grown hydroponically from bulbs subjected to two preparation procedures, 5 °C and 9 °C. Mean values ± Standard error; n = 10.

	Plant Leaf Area (cm^2/Plant)	Flower Stem Height (cm)	Flower Stem Diameter (mm)	Flower Stem Total FW (g/Flower Stem)	Flower Stem Total DW (g/Flower Stem)	Flower Bud DW (g)	Stem DW (g)	Leaf DW (g)	Bulb DW (g)
'Royal Virgin' (H1)									
5 °C	122.9 ± 34.4	40.7 ± 0.3	8.0 ± 0.6	18.4 ± 2.0	1.56 ± 0.08	0.40 ± 0.06	1.16 ± 0.06	0.96 ± 0.23	7.05 ± 0.94
9 °C	147.3 ± 22.3	42.2 ± 1.2	8.3 ± 0.9	20.9 ± 1.7	2.00 ± 0.15	0.40 ± 0.02	1.60 ± 0.13	1.11 ± 0.17	8.07 ± 1.15
'Ad Rem' (H2)									
5 °C	144.6 ± 40.6	31.5 ± 0.3	9.3 ± 0.9	17.9 ± 1.6	1.82 ± 0.27	0.43 ± 0.02	1.39 ± 0.27	0.91 ± 0.10	6.61 ± 0.96
9 °C	119.3 ± 5.9	43.0 ± 1.5	9.3 ± 0.1	20.2 ± 0.8	1.93 ± 0.03	0.53 ± 0.03	1.40 ± 0.02	0.84 ± 0.08	10.25 ± 0.10

Dry weight analysis reflects the results observed in plant biometric measurements and confirms that bulb-preparation procedure did not affect the matter partitioning in the different plant organs, with the exception of the dry matter accumulation in the stem in 'Royal Virgin', which was lower after bulb exposure to 5 °C (Table 1).

The preparation procedure of bulbs before planting did not affect the concentration of the main macronutrients (total nitrogen, phosphorus, and potassium) in tulip leaves and bulbs, with the exception of a reduction of K in 'Royal Virgin' after the exposure at 9 °C (Table 2).

Table 2. Concentration of the main macronutrients (g/100 g DM) in leaves and bulbs of the two hybrids of *Tulipa gesneriana* L. 'Royal Virgin' and 'Ad Rem', grown hydroponically from bulbs subjected to two preparation procedures, 5 °C and 9 °C, at the end of the growing cycle. Mean values ± Standard error; n = 3.

	Leaves			Bulb		
	N tot %	*p* %	K %	N tot %	*p* %	K %
'Royal Virgin' (H1)						
5 °C	3.17 ± 0.07	0.48 ± 0.08	18.03 ± 1.83	0.70 ± 0.07	0.07 ± 0.01	7.51 ± 0.50
9 °C	3.62 ± 0.53	0.61 ± 0.06	7.64 ± 1.32	0.71 ± 0.07	0.07 ± 0.01	7.15 ± 0.41
'Ad Rem' (H2)						
5 °C	2.79 ± 0.13	0.66 ± 0.01	17.36 ± 4.62	0.74 ± 0.19	0.11 ± 0.01	7.62 ± 0.52
9 °C	2.83 ± 0.07	0.62 ± 0.03	19.17 ± 1.60	1.03 ± 0.38	0.09 ± 0.01	6.52 ± 0.24

3.2. Analyzed Metabolites

Tulip plants 'Ad Rem' showed significant higher starch content compared with 'Royal Virgin' ones, only in leaves at flowering (on avg. 12.02 and 10.55 mg g^{-1} DW, respectively), while starch did not significantly differ in bulbs before planting and at flowering (on avg. 65.85 and 72.04 mg g^{-1} DW, respectively). However, the interaction *Hybrid* × *Preparation procedure* was significant for starch in bulbs before planting (Table 3). Sucrose content was not significantly affected by hybrids or by the preparation procedure in bulbs before planting, being on average 11.44 mg g^{-1} DW; meanwhile, hybrids and preparation procedures significantly influenced it in bulbs at flowering. In leaves at flowering, only the preparation procedure affected sucrose content (Table 3). Glucose and fructose contents were influenced by preparation procedure and hybrid, respectively, even if fructose underwent also a significant interaction between the two sources of variance in bulbs before planting. However, while fructose was not significantly affected by hybrid or cold treatment in bulbs and leaves at flowering, the interaction between the variables resulted significant for glucose in these different tissues at flowering. Chlorophyll a, chlorophyll b, and carotenoids did not significantly differ in dependence of hybrid, preparation procedure, or tissue. The interaction *Hybrid* × *Preparation procedure* was significant for polyphenols in bulbs before planting and leaves at flowering, while the type of hybrid affected polyphenols content in bulbs at flowering (on avg. 4.29 and 2.58 μg g^{-1} DW in 'Royal Virgin' and 'Ad Rem', respectively) (Table 3). Soluble proteins were significantly lower (−28%) in leaves of 'Royal Virgin' than in 'Ad Rem' ones at flowering, while they did not significantly differ in bulbs before planting and at flowering (Table 3).

Table 3. Soluble proteins, starch, glucose, fructose, sucrose, (in mg g^{-1} DW), chlorophyll a, chlorophyll b, carotenoids, and polyphenols (in μg g^{-1} DW) in bulbs and leaves of the two hybrids of *Tulipa gesneriana* L. 'Royal Virgin' and 'Ad Rem', grown hydroponically from bulbs subjected to two preparation procedures, 9 °C and 5 °C. ns—nonsignificant; *, ** and ***—significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Different lowercase or capital letters within each row, for specific vegetative stage, indicate significant differences ($p = 0.05$).

	'Royal Virgin' (H1)			'Ad Rem' (H2)			Significance		
Source of Variance	**5 °C**	**9 °C**	**Mean**	**5 °C**	**9 °C**	**Mean**	**Hybrid (H)**	**Preparation Procedure (P)**	**H × P**
Bulbs before planting									
Soluble proteins	46.7	43.2	*45*	43.7	43.9	*43.8*	ns	ns	ns
Glucose	37.94 a	35.44 b	*36.7*	37.85 a	33.38 a	*35.6*	ns	*	ns
Fructose	25.62 a	27.99 b	*26.8* A	29.66 ab	33.11 c	*31.4* B	**	ns	**
Sucrose	16.17 a	9.18 b	*12.67*	7.85 b	12.59 b	*10.22*	ns	*	ns
Starch	66.33 a	63.49 a	*64.9*	61.63 a	71.95 b	*66.8*	ns	ns	*
Polyphenols	0.92 a	0.98 a	*0.96*	0.98 a	0.91 b	*0.95*	ns	ns	ns
Bulbs at flowering									
Soluble proteins	43.47	42.43	*42.95*	39.94	45.79	*42.86*	ns	ns	ns
Glucose	31.01 a	37.88 b	*34.45*	36.30 ab	31.79 a	*34.05*	ns	ns	*
Fructose	30.66	36.07	*33.37*	31.48	29.12	*30.3*	ns	ns	ns
Sucrose	6.54 a	8.63 b	*7.59* A	8.08 a	11.94 b	*10.01* B	*	**	ns
Starch	68.82 a	76.71 b	*72.76*	68.48 a	74.19 b	*71.33*	ns	***	*
Polyphenols	4.46	4.12	*4.29* A	2.49	2.68	*2.58* B	***	ns	ns
Leaves at flowering									
Soluble proteins	62.49 a	48.87 ab	*55.68 A*	80.99 bc	73.63 c	*77.31 B*	*	ns	**
Glucose	66.49 a	73.91 ab	*70.2*	73.44 ab	87.18 b	*80.31*	ns	ns	*
Fructose	6.35	11.69	*9.02*	2.34	6.34	*4.34*	ns	ns	ns
Sucrose	0.99 a	1.12 a	*1.06*	0.65 a	2.71 b	*1.68*	ns	*	***
Starch	11.07	10.03	*10.55* A	11.49	12.54	*12.02* B	*	ns	ns
Polyphenols	3.57 a	5.26 b	*4.42*	5.67 b	4.09 b	*5.29*	ns	ns	**
Chlorophyll *a*	98.22	85.92	*92.07*	83.38	11.02	*97.20*	ns	ns	ns
Chlorophyll *b*	34.06	28.92	*31.49*	24.93	40.29	*32.61*	ns	ns	ns
Carotenoids	18.24	17.53	*23.37*	15.15	23.37	*19.26*	ns	ns	ns

The total free amino acids content (on avg. 117 μmol g^{-1} DW in bulbs of both hybrids before planting) was affected by the hybrid and the interaction between hybrid

and preparation procedure in bulbs before planting and leaves at flowering, while it was affected only by the interaction between the two sources of variance in bulbs at flowering (Table 4). The highest difference in total amino acids was found in leaves at flowering that strongly increased compared with the other two analysed tissues; moreover, there was a significant and strong difference between *Hybrids* × *Preparation procedure* (on avg. 537, 359, 205 and 242 µmol g^{-1} DW in H1 9°C, H1 5 °C, H2 9 °C and H2 5 °C, respectively). In bulbs before planting, glutamine, γ aminobutyric acid (GABA), proline, glutamate, threonine, and minor amino acids (the sum of arginine, isoleucine, histidine, leucine, lysine, methionine, phenylalanine, tyrosine, tryptophan, and valine) were the main amino acids, significantly higher than in bulbs at flowering (Table 4); whereas, in leaves at flowering, the values of amino acids on average 15-fold higher than in bulbs before planting, showed as main amino acids glutamine, alanine, serine, asparagine, minor amino acids, and branched chain amino acids (BCAAs). In particular, in leaves of H1 under 9 °C treatment, the highest amino acids values were found equal to 178, 116, 74.7, 65.0, and 47.3 µmol g^{-1} DW for glutamine, alanine, minor amino acids, serine, and asparagine, respectively (Table 4). Even if bulbs before planting showed lower values of amino acids compared with leaves at flowering, the amount of GABA, threonine, and proline were significantly higher in the former than in leaves (Table 4).

Table 4. Free amino acid content (µmol g^{-1} DW) in bulbs and leaves of the two hybrids of *Tulipa gesneriana* L. 'Royal Virgin' and 'Ad Rem' grown hydroponically from bulbs subjected to two preparation procedures, 9 °C and 5 °C. Alanine (Ala), asparagine (Asn), aspartate (Asp), γ-aminobutyric acid (GABA), glutamate (Glu), glutamine (Gln), Glycine (Gly), monoethanolamine (MEA), ornithine (Orn), proline (Pro), serine (Ser), threonine (thr), total amino acids (Total AA), minor amino acids (minor AA), and branched chain amino acids (BCAAs). ns—nonsignificant; *, **, and ***—significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively. Different lowercase or capital letters within each column, for specific vegetative stage, indicate significant differences ($p = 0.05$).

Source of Variance	Ala	Asn	Asp	GABA	Glu	Gln	Gly	MEA	Orn	Pro	Ser	Thr	Total AA	Minor AA	BCAAs
Bulbs before planting															
'Royal Virgin' (H1)															
5 °C	2.48 a	0.57 a	6.05	5.38 a	10.9 a	31.3 a	0.12 a	0.41 a	0.3 a	14.9	2.67	25.3 a	134 a	13.07	5.85
9 °C	2.73 a	0.7 a	6.59	31.6 b	15 b	30.4 a	0.12 a	0.41 a	0.27 a	18.3	2.75	29.5 a	126 a	14.09	6.27
Mean	*2.61* A	*0.64* A	*6.32* A	*15.5* A	*13*	*30.9* A	*0.12*	*0.41*	*0.29* A	*16.6*	*2.71*	*27.4* A	*130* A	*13.58*	*6.06*
'Ad Rem' (H2)															
5 °C	1.77 b	3.18 b	5.71	23.3 b	10.1 a	19.5 b	0.56 b	0.43 a	0.2 b	18.6	2.68	3.21 b	103 b	13.44	6.61
9 °C	2.24 a	2.84 b	4.93	23.8 b	14.3 b	22.4 b	0.1 a	0.31 b	0.25 ab	14.9	3.16	5.66 b	108 b	12.52	5.87
Mean	*2.01* B	*3.01* B	*5.32* B	*23.6* B	*12.2*	*20.9* B	*0.33*	*0.37*	*0.22* B	*16.7*	*2.92*	*4.43* B	*105* B	*12.98*	*6.24*
Hybrid (H)	**	**	*	*	ns	*	ns	ns	**	ns	ns	**	***	ns	ns
Preparation procedure (P)	ns	ns	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
H × P	*	*	ns	**	ns	**	*	*	*	ns	ns	***	**	ns	ns
Bulbs at flowering															
'Royal Virgin' (H1)															
5 °C	0.99 a	1.26 a	2.07	5.05	3.22 b	5.69 a	0.19 a	0.33	0.32 a	2.97 a	0.99 a	1.64 a	30.4 a	5.35 a	2.46
9 °C	0.79 ab	0.94 a	2.04	5.38	5.91 a	4.27 b	0.19 a	0.3	0.48 b	4.23 ab	0.91 b	7.81 ab	65.4 b	5.42 a	2.49
Mean	*0.89* A	*1.1*	*2.05*	*5.21*	*4.57*	*4.98* A	*0.19*	*0.32*	*0.4*	*3.6*	*0.95*	*4.72*	*47.9*	*5.39*	*2.47*
'Ad Rem' (H2)															
5 °C	0.74 b	2.59 b	2.41	5.37	3.19 b	2.47 c	0.22 a	0.43	0.33 a	4.94 b	1.18 a	2.78 ab	35.3 a	8.35 b	2.76
9 °C	0.59 b	1.46 a	2.19	5.72	3.14 b	2.45 c	0.07 b	0.35	0.3 a	2.95 a	0.73 b	4.72 b	30.8 a	5.82 a	2.16
Mean	*0.66* B	*2.02* B	*2.3*	*5.55*	*3.16*	*2.46* B	*0.15*	*0.39*	*0.32*	*3.94*	*0.95*	*3.75*	*33.1*	*7.09*	*2.46*
Hybrid (H)	*	*	ns	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	ns
Preparation procedure (P)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	**	ns	ns	ns
H × P	*	*	ns	ns	*	***	***	ns	***	*	ns	***	*	*	ns
Leaves at flowering															
'Royal Virgin' (H1)															
5 °C	79 a	32.3	6.16	1.09	4.91 a	126 a	4.99 ab	1.57	0.44 a	12.9 a	38.7 a	2.7 a	359 a	47.35 a	18.4 a
9 °C	116 b	47.3	7.3	1.13	9.29 b	178 b	9.13 a	2.14	0.53 a	18.3 b	65 a	7.9 b	537 b	74.73 b	28.2 b
Mean	*97.5* A	*39.8*	*6.73*	*1.11*	*7.1*	*152* A	*7.06* A	*1.86*	*0.48*	*15.6* A	*51.8* A	*5.3* A	*448* A	*61.04* A	*23.3* A
'Ad Rem' (H2)															
5 °C	35.3 c	34.7	6.55	1.11	5.64 a	82.5 a	5.61 ab	1.57	0.76 b	6.16 c	24.6 b	2.29 a	242 c	34.51 a	14 a
9 °C	29.9 c	28.5	5.72	0.85	4.02 a	73.4 c	2.94 b	1.43	0.4 a	6.42 c	20.3 c	1.3 a	205 d	29.5 a	13.5 a
Mean	*32.6* B	*31.6*	*6.13*	*0.98*	*4.83*	*77.9* B	*4.28* B	*1.5*	*0.58*	*6.29* B	*22.5* B	*1.8* B	*224* B	*32.01* B	*13.8* B
Hybrid (H)	**	ns	ns	ns	ns	**	*	ns	ns	**	**	*	**	**	**
Preparation procedure (P)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
H × P	***	ns	ns	ns	**	*	*	ns	***	***	*	***	*	**	**

3.3. Heat Map and Principal and Component Analysis of the Analysed Biochemical Parameters

An in-depth overview of the morphophysiological and biochemical changes of the two tulip hybrids under the different cold treatments was obtained by conducting a heat map and a principal component analysis (PCA) for all the measured parameters. These

types of multivariate analyses may allow the identification of phenotypic variation patterns associated with genotype and/or preparation conditions or procedures, as previously shown in Ranunculus [22–24]. The data of chlorophylls and carotenoids, measured only in the leaves, were not included in these analyses. This choice was made because, if these data had been included, it would have been impossible to compare the metabolism changes in bulbs and leaves at the same time. Moreover, their differences were not significant, demonstrating that these parameters were unaffected by different preparation procedures.

The aggregated data heat map analysis identified two main clusters, corresponding to the leaf samples at flowering (af) in the upper left part of the figure, and bulb samples at flowering (af) and before planting (bp) on the lower left part (Figure 1). In the bulbs before planting, the levels of free amino acids were lower than in the leaves, with the exception of GABA, proline, glutamate, aspartate, and threonine (that were present at higher levels in H1). However, fructose, sucrose, and starch were much higher in the bulbs than in the leaves. In particular, in bulbs of 'Royal Virgin' at 5 °C, threonine and sucrose were accumulated, while in the same hybrid pretreated at 9 °C, threonine, proline, and glutamate showed higher contents (Figure 1). In the 'Ad Rem' bulbs, threonine was not present, but, while the 9 °C treatment showed accumulation of glutamate and sucrose, in addition to GABA, the 5 °C treatment showed proline and GABA but not sucrose, whereas polyphenols were much less concentrated in the bulbs before planting than in the other samples. The leaves, in particular those of H1, showed high levels of soluble proteins, amino acids, polyphenols, and glucose.

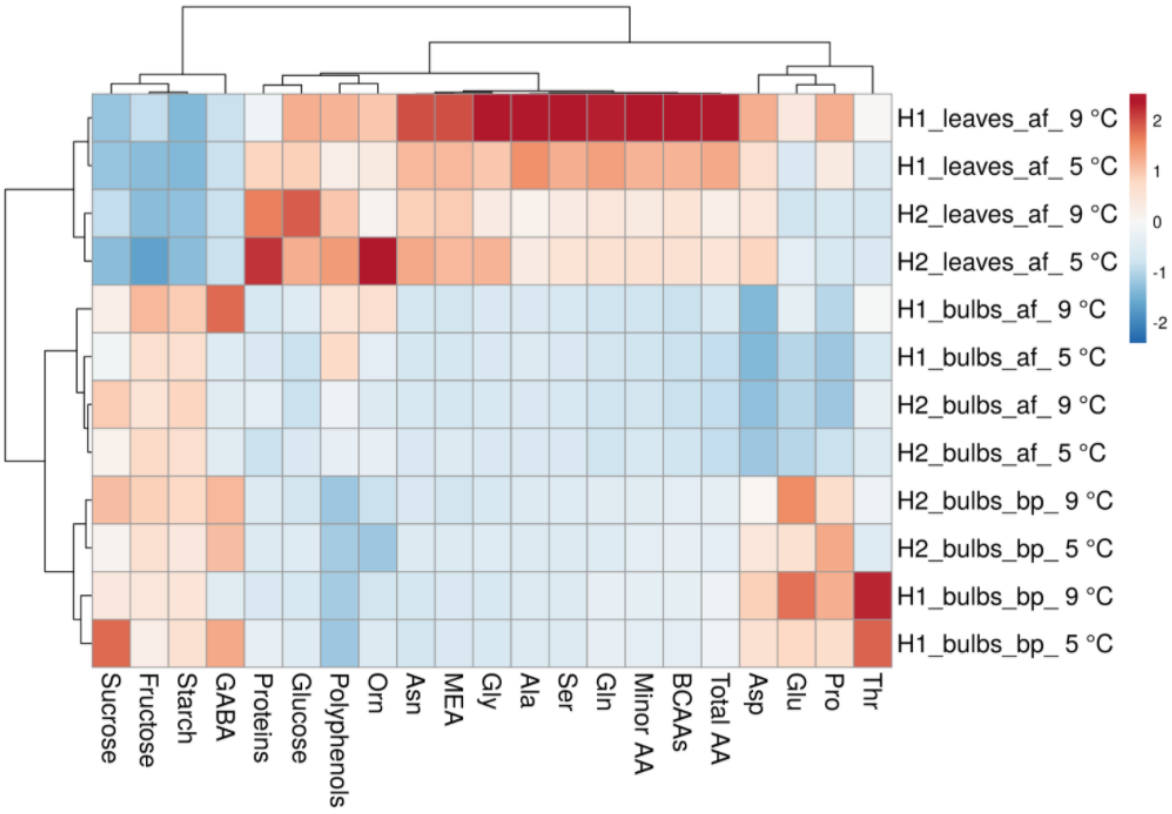

Figure 1. Cluster heat map analysis summarizing the changes in the metabolic profile of bulbs before planting and at flowering and leaves at flowering of the two hybrids of Tulipa gesneriana L. 'Royal Virgin' and 'Ad Rem', grown hydroponically from bulbs subjected to two preparation procedures, 5 °C and 9 °C. The cluster heat map was generated using the https://biit.cs.ut.ee/clustvis/ (accessed on 12 March 2022) online program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage.

The PCA of analysed biochemical parameters highlighted that the first two principal components (PCs) were related with eigen values higher than 1 and explained 85.1% of the total variance, with PC1 and PC2 accounting for 66.7% and 18.4%, respectively. The score plot of the PCA separated the bulbs and leaves along PC1 and the different hybrids x temperature treatment along PC2 (Figure 2). Leaves at flowering were concentrated in positive side of PC1 with H1 and H2 in the upper and lower right quadrants, respectively. Bulbs before planting and at flowering were clustered in the negative side of PC1 in the upper and lower left quadrants, respectively. PC1 was positively related to total and minor amino acids, and in particular to asparagine, glycine, branched chain amino acids (BCAAs), glutamine, serine, alanine, and glucose. PC1 was negatively correlated with starch, fructose, sucrose, and GABA. PC2 was positively correlated with proline, glutamate, threonine, and aspartate; while it was negatively correlated to polyphenols (Figure 2).

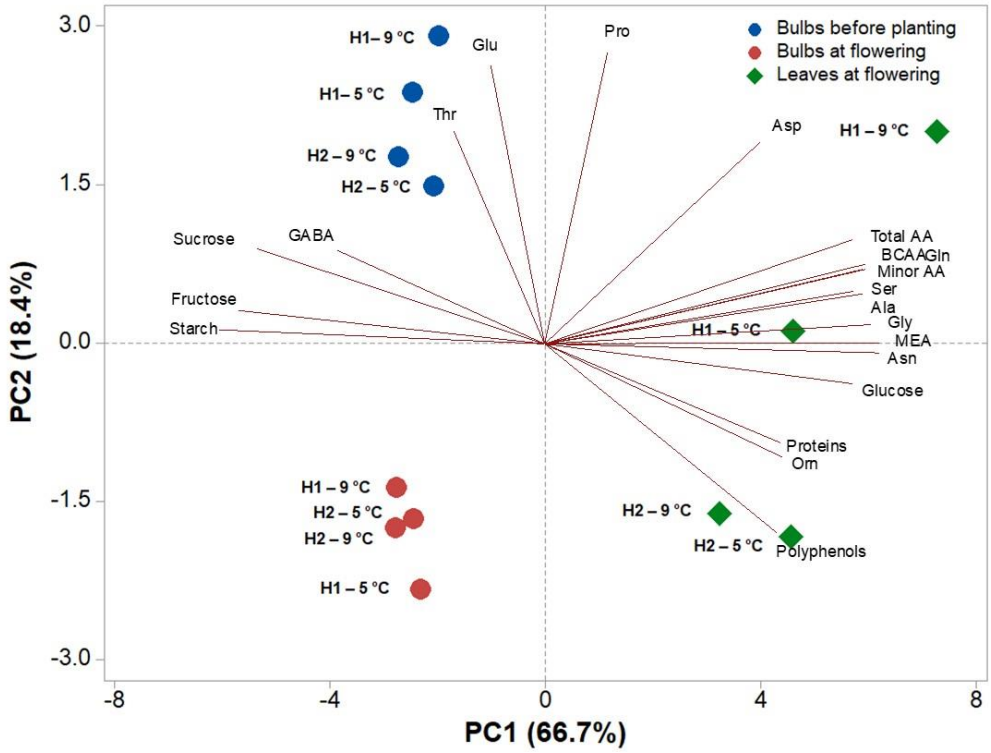

Figure 2. Principal component loading plot and scores of principal component analysis (PCA) of metabolic profile parameters of bulbs and leaves of tulip hybrids 'Royal Virgin' and 'Ad Rem', grown hydroponically from bulbs subjected to two preparation procedures, 5 °C and 9 °C.

4. Discussion

Plants of tulip (*Tulipa gesneriana* L.) hybrids 'Royal Virgin' and 'Ad Rem' showed a good adaptation to the hydroponic environment, developing healthy roots of a white colour, with no brown area on the apexes, and intense green leaves, with no symptoms of nutrient deficiency, and reached the typical size in terms of both leaf area and flower stem known for the two genotypes.

The time for the beginning of flowering in the cultivation conditions applied in the experiment was similar in the two genotypes, and the duration of flowering period was very short, as expected in clones of tulips propagated by bulbs [12].

The preparation procedure did not influence the flowering earliness in 'Royal Virgin', while the cold treatment at 9 °C slightly anticipated flowering compared with that at 5 °C in 'Ad Rem'. This result highlights that both the combinations of low temperature and time of exposure fulfilled the cold requirement of the tulip genotypes; however, it also confirms the genotype-specific response known for this crop [9]. In this respect, in

plants of the hybrid 'Ad Rem', it is possible that lower preparation temperatures determine stress conditions with negative effects on the flowering process, as proved by the significant increase in metabolites previously found involved in cold stress and/or acclimation, such as threonine, sucrose, proline, or GABA [25–27] (Figure 1). Proline and GABA may be rapidly accumulated for cell protection against stress, mainly as ROS scavengers for stabilizing membranes and macromolecules against oxidative stress, and then broken down upon relief of stress to supply carbon, nitrogen, and energy to recover and repair stress-induced damages [25]. Moreover, the synthesis of GABA is a proton-consuming reaction able to cope with the cytosolic acidosis that can happen in response to an abrupt transfer to lower temperature [28]. Highly increased sucrose, together with threonine, in particular in H1 bulbs pretreated at 5 °C, in addition to having a protective role against cold stress being also indirectly helpful as a substrate for metabolism, can directly regulate cold acclimation by mediating an increase in GUS activity, as a reporter for the activity of the cold-responsive COR78 promoter in Arabidopsis [29]. Probably, the incapacity of H2 bulbs pretreated at 5 °C to accumulate sucrose determined a lower cold stress tolerance and a delay in flowering. Regarding threonine, Klein et al. [30] and Yuan et al. [31] have both explained that its increase under cold acclimation could depend on its function as precursor of the aspartate-derived amino acid pathway, in particular for the synthesis of branched chain amino acids (BCAAs). BCAAs may serve as substrate for the synthesis of stress-induced proteins, or act as signalling molecules to regulate stress-related gene expression [32]. BCAAs may also play a role as ROS scavengers through a not fully elucidated mechanism and/or function as alternative electron donors for the mitochondrial electron transport chain to allow plant growth after stress relief [33]. In H2 bulbs pretreated at 9 °C, the symptoms of cold stress were perceived as milder, as shown by the lower proline and threonine levels than in other bulbs before planting (Figure 1).

As expected, the two hybrids formed plants with different characteristics and a different response to the two preparation procedures, also in terms of flower stems characteristics. Particularly, while in 'Royal Virgin' the thermal history of propagation material did not influence biometric parameters of cut flowers, in 'Ad Rem', treatment at 9 °C increased the stem height compared with those at 5 °C, with a consequent increase in fresh weight at the harvest. This is in accordance with other evidence which has shown different cold requirements and different flowering responses in tulip cultivars [12]. Indeed, in this response, the initial lower stress perceived by 'Ad Rem' bulbs treated at 9 °C allowed them to invest lower energy in stress defence metabolites, using them for growth.

The metabolism of amino acids and proteins appeared particularly active in the leaves at flowering but differently in the two hybrids. While 'Royal Virgin' leaves, and in particular those pretreated at 9 °C, showed high quantities of total and minor amino acids, among which proline, 'Ad Rem' showed high levels of proteins. This means that, while 'Royal Virgin' plants were still recovering from cold stress, 'Ad Rem', in particular the samples pretreated at 9 °C, was able to invest more in plant biomass. The high quantities of polyphenols and glucose in leaves also confirms the presence of a still-active photosynthesis, able to synthesize carbon skeletons to be exported to the bulbs for conversion into starch and fructose, which were accumulated in both types of bulbs independently of thermal pretreatment.

5. Conclusions

In conclusion, our results highlight that the plant response to the cold treatment of tulip bulbs is a genotype-dependent response. The thermal level and the time of exposure influence the plant metabolism and growth and can modify flowering earliness and flower characteristics. Based on this evidence, preliminary study to evaluate the effects of different preparation procedures on the different genotypes would help breeders to optimize plant performance and production scheduling in commercial farms.

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