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Changing in the production of anticancer drugs (vinblastine and vincristine) in *Catharanthus roseus* (L.) G. Don by potassium and ascorbic acid treatments

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Abstract: *Catharanthus roseus* seedling was treated with different concentrations (1.5, 3.16, 15, and 30 mmol) and forms (K_2SO_4 and KNO_3) of potassium (K^+) *via* Hoagland's nutrient solution. Ascorbic acid (AsA) was sprayed twice (plant days 68 and 78) with different concentrations (750 and 1 500 mg/L) on the leaves. Vinblastine, vincristine, tryptophan contents, *D4H* and *DAT* genes expression, peroxidase activity, and H_2O_2 content of leaves were measured. Potassium in KNO_3 form increased vinblastine (60%) and vincristine (50%), compared to 30% and 20% using K_2SO_4. Vinblastine and vincristine inhibit microtubule assembly and ultimately metaphase-arrested caused by the polymerisation. The genes expression was higher 3 times in KNO_3 and 2.5 times in K_2SO_4 in excess of K^+. Foliar application of 750 mg/L AsA led to an increase in vinblastine (20%) and vincristine (16%). Both concentrations of AsA had the same additional effect on the expression of *D4H* and *DAT* about 30% and 60%, respectively, compared to the control plant. Tryptophan decreased 2.5 times in excess of K^+ and 35% due to the exterior of AsA. H_2O_2 decreased while peroxidase activity increased along with AsA treatment. A positive interaction existed between the K^+ and AsA on the amount of vinblastine, vincristine, tryptophan, and gene expression.

Keywords: terpenoid indole alkaloids; anticancer drugs production; secondary metabolites pathway; medicinal plant; nutrient treatment; antioxidant treatment

Because plants are a rich source of bioactive compounds, since the pre-history era, the therapeutic properties of plants to treat diseases have been used. According to different mechanisms of the therapeutic metabolites of plants, researchers investigated their anti-cancer effects to treat various cancers.

Alkaloids are one of the largest groups of secondary metabolites in plants. Alkaloids belong to the glycosides and are of high value as anti-cancer compounds. There are three primary groups of alkaloids. The first group is indole alkaloids such as vinblastine and vincristine from *Catharanthus roseus*. Quinoline alkaloid such as camptothecin from *Camptotheca acuminate* is the second group. The last group is diterpenoid alkaloids such as taxol, and it's analogous to Taxus and Corylus species.

Catharanthus roseus (L.) G. Don, Madagascar periwinkle, is a medicinal plant that belongs to the Apocynaceae family, originated from Madagascar. This plant is cultivated in different parts of the world. Also, *C. roseus* produces terpenoid indole alkaloids (TIAs) for use in the pharmaceutical industry. Vinblastine and vincristine as dimeric indole alkaloids and as effective cancer drugs are extracted from the leaves of *C. roseus*.

In cell biology, vinblastine and vincristine bind to tubulin and cause polymerisation. This polymerisation results in inhibiting microtubule assembly. This inhibition leads to the disruption in the mitotic spindle, ultimately metaphase-arrested (Zandi 2021). Microtubule poisoning caused by vinblastine and vincristine triggers spindle assembly checkpoint

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proteins. Subsequently, it halts cell cycle progression to permit spindle readjustment and prevent aberrant chromosome segregation. Mitotic catastrophe, a cell death mechanism mediated by aberrant mitosis, was happened by the impaired mitotic assembly checkpoint proteins and abnormal chromosome segregation (Bagnyukova et al. 2010).

C. roseus always produces TIAs in the cell and tissue culture, but these *in vitro* methods are inadequate to satisfy the requirements of mass production. Interestingly, vinblastine was not detected in all cultures at the end of the second week of the experiment (Hemmati et al. 2020). Due to cell division and longitudinal growth, *in vitro* cultures growth continues for a short time, and stops because of the accumulation of toxic substances and the termination of nutrients within the medium (Bhojwani and Razdan 1986). Thus, the short phase of *in vitro* tissue differentiation inhibiting the activity of the present and active enzymes in differentiated or mature plants is a predictable reason to produce lower secondary metabolites in cell and plant tissue culture (Dias et al. 2016). Though the relative profusion of specific alkaloids was more in some petals than others, the second distribution of alkaloids in different petals of the same flower was entirely compatible (Dutkiewicz et al. 2020). Based on recent studies, cell-specific synthesis of alkaloids in stem and leaf of *C. roseus* results in low biosynthesis of vinblastine (Roepke et al. 2010, Yamamoto et al. 2019). The lack of knowledge on the biosynthesis of secondary metabolites and how they are regulated is another main reason to justify the low performance of these compounds (Verpoorte et al. 2002). Therefore, plant cultivation can be the only commercial way to produce vinblastine and vincristine (Loraine and Mendoza-Espinoza 2010).

Potassium (K^+) is an essential cation for plant growth, biochemical processes, enzymatic activation, and transcription in various pathways (Devi et al. 2012). Also, K^+ transport is critical to control cytoplasmic and luminal pH in endosomes, regulation of membrane potential, and enzyme activity. Plants have evolved a large group of K^+ transporters with defined functions in nutrient uptake by roots, storage in vacuoles, and ion translocation between tissues and organs. Plant cells have developed unique transport systems for K^+ accumulation and release. Potassium channels played an important role in potassium uptake and efflux; hence, they are considered to be one of the best-characterised classes of membrane proteins in plants. Uptake and allocation of K^+ are performed by K^+ channels and transporters belonging to different protein families with varied structures and transport mechanisms. These families are the channel families Shaker-like voltage-dependent, the tandem-pore, the two-pore channels (Hedrich 2012), the carrier-like families KT/HAK/KUP (Nieves-Cordones et al. 2014, Li et al. 2018), high-affinity potassium transporter (HKT) (Hamamoto et al. 2015), and cation-proton antiporters (Ragel et al. 2019).

Ascorbic acid (AsA) is a water-soluble antioxidant molecule that has multiple developmental roles in plants. For instance, AsA enables plants to withstand stresses and various abiotic and biotic conditions. AsA contributes as an enzyme cofactor and precursor in the production of primary and secondary metabolites (Herrera-Martínez et al. 2013). In the TIAs pathway, *d4h* (EC1.14.11.20) is an oxidoreductase enzyme, and AsA acts as an activator (www.brenda-enzymes.org), cofactor (enzyme.expasy.org), and an H^+ donor for the maximal activity for this enzyme (www.brenda-enzymes.org). The effect of AsA as a bio-fertiliser in *Vicia faba* L. (Mohsen et al. 2013) and foliar application in *Chenopodium quinoa* Wild. (Aziz et al. 2017) has been already determined. Their results indicated that the addition of AsA resulted in a significant increase in the growth parameters. Also, the effect of AsA on gene expression in *Arabidopsis thaliana* (L.) Heynh. has been reported (Qian et al. 2014).

There is little information related to the dual effect of K^+ and AsA on the physiological responses of *C. roseus*. However, positive interaction between the K^+ fertilisation and foliar spray of AsA was reported for sweet potato (*Ipomoea batatas* (L.) Lam.). The highest value of growth characters and the total yield were observed in the combination of K^+ and AsA in sweet potato (El-Seifi et al. 2014). El-Morsy reported the interactions between K^+ and antioxidants (especially AsA) on total yield in garlic (*Allium sativum* L.) (El-Morsy et al. 2010).

C. roseus produces vinblastine and vincristine used to treat cancers (Loraine and Mendoza-Espinoza 2010). The failure of the industrial sectors to produce these medicinal compounds motivates researchers to find a new way to increase the amount of the alkaloids in *C. roseus*. Evaluating the effect of different concentrations of plant nutrients on the alkaloid pathway is one of the best practical and inexpensive methods to increase the synthesis of these compounds. Regarding the effect of K^+ and AsA on secondary metabolites, it could be predicted that K^+, AsA, and their interaction may affect alkaloids

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and tryptophan content in *C. roseus*. Therefore, the present research was conducted to evaluate the effect of different concentrations of K^+ and/or AsA on, (a) physiological factors such as peroxidase activity and H_2O_2 content, which could affect vinblastine and vincristine biosynthesis pathway; (b) tryptophan as a precursor of alkaloids; (c) desacetoxyvindoline-4-hydroxylase (*D4H*) and deacetylvindoline 4-O-acetyl transferase *(DAT*) genes expression as the key genes at the end of biosynthesis pathway of alkaloids in *C. roseus*, because desacetoxyvindoline-4-hydroxylase (d4h) and deacetylvindoline 4-O-acetyl transferase (dat) enzymes are rate-limiting enzymes (Liu et al. 2021), and in young leaves, it is closely related to the expression of *D4H* and *DAT* genes (El-Sayed and Verpoorte 2007), and (d) vinblastine and vincristine as important alkaloids.

MATERIAL AND METHODS

Plant materials and treatments. *C. roseus* seeds were prepared from the Medicinal Plant Research Center, Isfahan, Iran. Sterilised seeds germinated in Petri dishes using a water agar culture medium. Uniform 3-day-old seedlings were transferred to pots filled with perlite and irrigated with half-strength Hoagland's nutrient solution. They were kept in the growth chamber of the University of Isfahan at 28 °C and 65% relative humidity under a 16/8 h (light/dark) photoperiod. Potassium was added to Hoagland's nutrient solution after the appearance of four leaves in the plants (one month after germination) in such a way that the nutrient solution contained 1.5 mmol K^+ (as insufficient), 3.16 mmol (as control, recommended concentration in Hoagland's nutrient solution), 15 and 30 mmol (as an excess of K^+). Using two forms of potassium (K_2SO_4 and KNO_3) showed the effect of K^+ in conjunction with sulfate and nitrate. AsA spraying manually took place twice on the surface of leaves with the concentrations of 0 (distilled water, as control), 750 and 1 500 mg/L, containing 0.1% Tween 20 as the surfactant agent. The plants were sprayed twice, the first time when the age of the plants was 68 days and the second time when the age of the plants was 78 days (growth phase diminishing time). The plant samples were collected at the end of the growing season when the plants were 90 days old (before the flowering phase), transferred to the liquid nitrogen, and kept at –80 °C in the refrigerator.

Sample preparation and HPLC analysis of vinblastine, vincristine and tryptophan. Vinblastine and vincristine of *C. roseus* leaves were measured (Sajadi and Verpoorte 2000, Hisiger and Jolicoeur 2007). Briefly, the frozen leaves of the plants were lyophilised and 0.2 g of the dry leaves powder homogenised for 5 min by an ultra-homogeniser (Heidolph, Silent Crusher M, Schwabach, Germany) with 10 mL methanol containing 2% formaldehyde at 10 000 *g* in an ice bath. After incubation for 2 h, the samples were centrifuged at 13 000 *g* for 10 min. Supernatants were separated and dried completely using a rotary evaporator at 40 °C at reduced pressure (0.40 bar). Then, 14% ammonia (1 mL) was added to unionise the alkaloids and increase their hydrophobicity. Alkaloids were back-extracted with diethyl ether (1 mL) seven times. After drying the residual aqueous part on Na_2SO_4, it was solubilised in HPLC solvents A and B (1:1) by 1 mL before HPLC analysis.

HPLC analysis. HPLC instrument 1: Solvent delivery unit (LC-30AD), UV-Visible detector (SPD-20A, Tokyo, Japan), autosampler (SIL-30AC, Tokyo, Japan) oven (CTO-20AC, Tokyo, Japan), reversed-phase Phenyl HPLC column (250 mm × 3 mm, 5 µm particle size, Waters, USA), and LabSolution software (Tokyo, Japan). HPLC instrument 2: Knauer pump 6.1 L (Azura, Berlin, Germany), Shimadzu prominence RF-20A fluorescence detector, Clarity software, and C18 reversed-phase column (150 × 4.6 mm, 5 µm) by Rheodyne injector fitted with a sample loop. We used HPLC instrument 1 to determine vinblastine and vincristine. UV-visible wavelength was 280 nm, the sample and standard volume was 40 µL, and the column oven temperature was 45 °C. Mobile phase A was acetonitrile:methoxy ethanol:buffer solution (15:5:80). Mobile phase B was acetonitrile:methoxy ethanol:buffer solution (45:5:50). The buffer solution was prepared from NaH_2PO_4 40 mmol (3.12 g/L) by adding Na_2HPO_4 (until pH 6.5). Also, we used HPLC instrument 2 to determine tryptophan. Emission and excitation wavelengths were 450 nm and 430 nm, and the sample volume was 20 µL. Mobile phase A: 1.02 g potassium dihydrogen phosphate and 0.435 g dipotassium hydrogen phosphate were dissolved in 500 mL ultrapure water. Mobile phase B: 75 mL ultrapure water, 225 mL acetonitrile, and 200 mL methanol. The flow rate and column oven temperature were 1.3 mL/min and 30 °C (Salmanizadeh and Sahi 2020). Vinblastine, vincristine, tryptophan standards, methanol, ammonia, diethyl ether, acetonitrile, methoxy ethanol, and ultrapure water were purchased from Sigma Aldrich (Burlington, USA).

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RNA extraction and real-time quantitative PCR. 100 mg fresh leaves samples were used to extract total RNA by Trizol reagent (YTzol Pure RNA) and were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). All RNA isolates had an OD_{260}:OD_{280} between 1.8 and 2.0, and the red safe stained test agarose-gel electrophoresis was used to verify the quality. Then, cDNA was synthesised from total RNA using QuantiTect Qiagen Kit according to the manufacturer's instructions with oligo (dT) as the primer. QRT-PCR analysis was done using cDNA and gene-specific primers with an Ampliqon SYBR. The primers used for the real-time PCR are:
D4H–F: 5'–TTGGGACAAGCAAGCACTCA–3'; D4H–R: 5'–GCTCCAGGAATGAAGGGGAC–3'; DAT–F: 5'–TTCCCTCCGGAAGCCATAGA–3'; DAT–R: 5'-GCTGATTTCCCTGCTACCGT–3'; CrActin–F: 5'–CTATGTTCCCAGGTATTGCAGATAGA–3'; CrActin–R: 5'–GCTGCTTGGAGCCAAAGC–3'

PCR. PCR was performed at 94 °C for 5 min, at 94 °C for 30 s, at 60 °C for 30 s, at 72 °C for 30 s and 80 °C reading sample in the rotor for 1 s for 35 cycles (Rotor-Gene Q 5plex (Qiagen)). The *Actin* housekeeping gene was used as an internal control. Reactions were repeated three times for each sample to ensure the reproducibility of the results. After the PCR reaction, a melting curve was obtained by Opticon version 3 (Hercules, USA). Parameters were set as a reading sample every 0.5 °C incensement in the range of 60–95 °C. The comparative cycle threshold method was used to analyse the relative transcript levels of *D4H* and *DAT* for different treatments (Livak and Schmittgen 2001).

Determination of peroxidase (POD) activity. 1 g fresh leaves were homogenised in 5 mL of Na-phosphate buffer (100 mmol, pH 7.8), containing EDTA (1 mmol), dithiothreitol (DTT, 1 mmol) and polyvinylpyrrolidone (PVP, 2% *w*/*v*). The homogenates were centrifuged at 13 000 *g* for 20 min at 4 °C. The supernatant was used for the assay of POD enzyme activity. The assay mixture was 1.99 mL of 50 mmol sodium phosphate buffer (pH 7.0), consisting of 0.1 µmol EDTA, 10 mmol guaiacol, 15 mmol H_2O_2, and then 100 µL of the enzyme extract was added to obtain a volume of 2 mL. The formation of tetraguaiacol from guaiacol was determined at 470 nm. The activity of POD was calculated by the extinction coefficient of tetraguaiacol (26.6 mmol/cm) (Plewa et al. 1991).

Determination of H_2O_2 content. 500 mg fresh leaves were homogenised with trichloroacetic acid 0.1% (*w*/*v*) in the dark on ice. The homogenate was centrifuged at 12 000 *g* for 15 min at 4 °C. 0.5 mL of supernatant from each tube was placed in a new tube, and 0.5 mL of potassium phosphate buffer 10 mmol (pH: 7) and 1 mL potassium iodide (KI: 1 mol) were added to it. After incubation of samples for 15 min at room temperature, the absorbance was read at 390 nm using a spectrophotometer (Shimadzu, Japan). H_2O_2 was determined using a standard curve (Velikova et al. 2000).

Statistical analysis. The experiments were performed as a factorial layout in a completely random design with three replicates. Statistical analysis was performed using SPSS 22 (Chicago, USA). The Duncan multiple range test was used for mean comparisons, and Excel (Washington, USA) was used to draw the figures.

RESULTS

Amount of vinblastine and vincristine. The K^+ amount and forms had a significant effect on vinblastine and vincristine of *C. roseus* leaves. Their amounts increased with an excess of K^+ while decreased in K^+ deficiency. The highest amount of vinblastine and vincristine was measured in 30 mmol K^+ (in KNO_3 form), which increased 60% and 50%, respectively, compared to the control plant. The plants treated with the same amount of K^+ in the form of K_2SO_4 showed lower vinblastine and vincristine growth, 30% and 20%, respectively. The deficiency of K^+ caused to reduce almost 30% of the amount of both alkaloids compared to the control plant.

Using 750 mg/L AsA at the optimum concentration of K^+ caused an increase in the amount of vinblastine and vincristine, almost 20% and 16%, respectively, compared to the control plant. Although the use of 1 500 mg/L AsA increased the content of vinblastine and vincristine by less than 750 mg/L, their amounts were higher than the control plants (3.16 mmol K^+ without AsA).

An interaction existed for the effect of KNO_3 and K_2SO_4 on the alkaloids content. Using 1 500 mg/L AsA and excess of K^+ increased vinblastine by approximately 60% using KNO_3 rather than 30% in the form of K_2SO_4 compared to control plants. The amount of vincristine was 50% and 20% higher using KNO_3 or K_2SO_4, respectively, in the same condition. 750 mg/L AsA with 1.5 mmol K^+ offset the deficiency of K^+. Consequently, the amount of vinblastine and vincristine was the same as the control plants (Figure 1).

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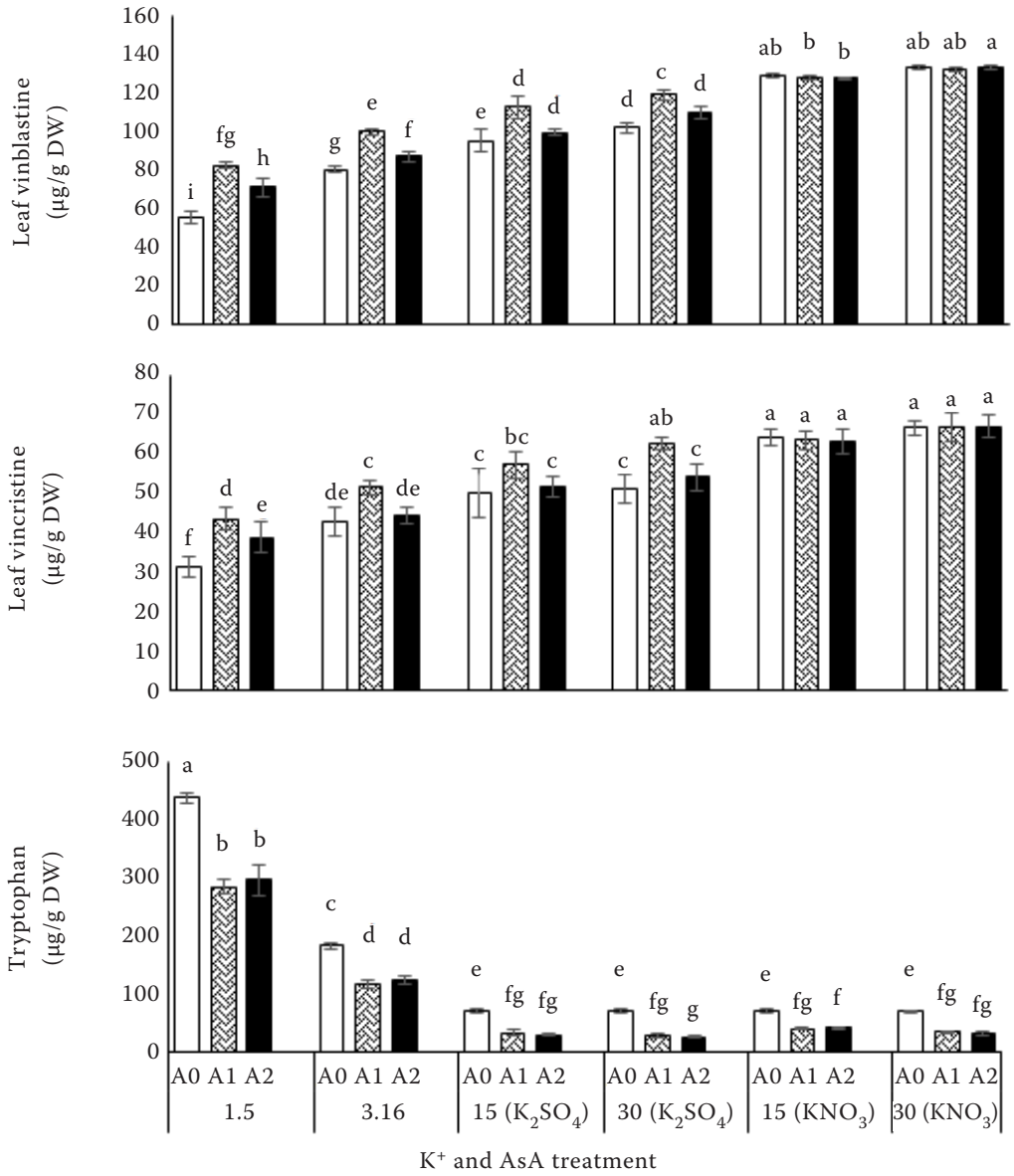

Figure 1. Vinblastine, vincristine, and tryptophan content (µg/g dry weight (DW)) in the leaf of 90-day old *Catharanthus roseus*. Abbreviated letters A0, A1, and A2 used for 0 (control), 750, and 1 500 mg/L ascorbic acid (AsA), respectively. Potassium (K^+) concentrations (mmol) (1.5: deficiency, 3.16: control, 15 and 30: excess) used as either KNO_3 or K_2SO_4. The values are the means of three independent biological replicates for each treatment. The bar lines represent the standard deviation and the different letters indicate the significant differences ($P \leq 0.05$) between different groups of plants based on Duncan's multiple range test

Amount of tryptophan. Two forms of K^+ (KNO_3 and K_2SO_4) did not affect tryptophan content, but its amount was inversely correlated with the K^+ amount. The amount of tryptophan was two times higher in K^+ deficiency and 2.5 times lower in excess of K^+ compared to the control plants.

AsA acted the same as K^+ did to amino acids and reduced its amount as the AsA amount increased. Although AsA had a significant effect ($P \leq 0.05$) on the amount of tryptophan, different concentrations of AsA (750 and 1 500 mg/L) did not affect tryptophan content. Using 750 or 1 500 mg/L sprays of AsA in K^+ deficiency reduced tryptophan content by 35%.

The dual effect of K^+ (both forms) and AsA had a significant interaction ($P \leq 0.05$) on the amount of tryptophan. The lowest amount of tryptophan was observed in the combination of K^+ and AsA. Tryptophan content decreased by almost 80% in the combined increase of K^+

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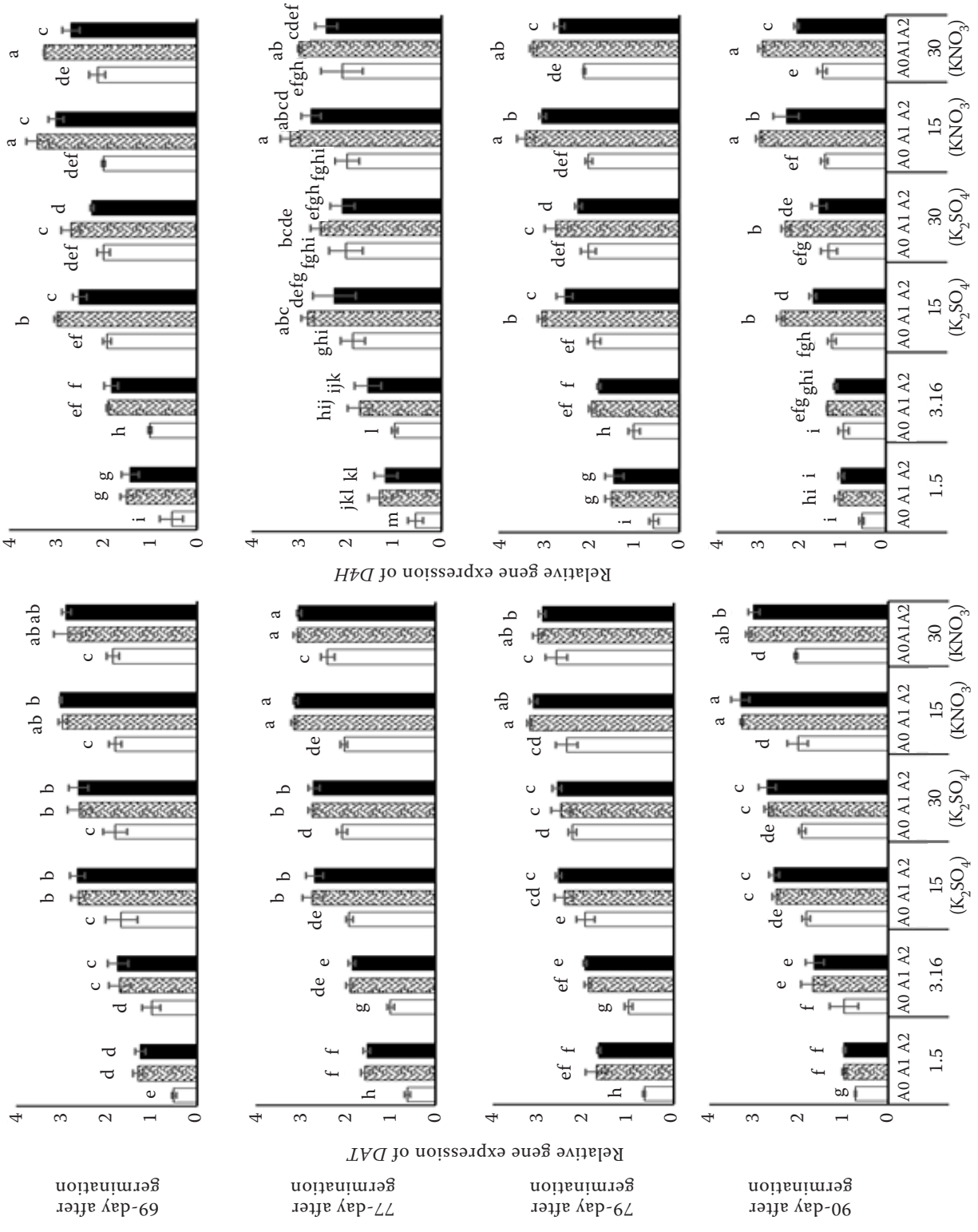
Figure 2. Relative expression of *D4H* and *DAT* in the leaves of *Catharanthus roseus* plant. Abbreviated letters A0, A1, and A2 used for 0 (control), 750, and 1 500 mg/L ascorbic acid , respectively. Potassium concentrations (mmol) (1.5: deficiency, 3.16: control, 15 and 30: excess) used as either KNO_3 or K_2SO_4. The values are the means of three independent biological replicates for each treatment. The bar lines represent the standard deviation and the different letters indicate the significant differences ($P \leq 0.05$) between different groups of plants based on Duncan's multiple range test



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and AsA. The highest amount of tryptophan was observed in deficiency of K^+ and no AsA. It was an almost 90% decrease in tryptophan content between K^+ deficiency with no AsA and excess of K^+ and AsA (Figure 1).

***D4H* and *DAT* genes expression.** There were no differences between KNO_3 and K_2SO_4 in the expression of *D4H* and *DAT*. However, the concentrations of K^+ showed a significant difference ($P \leq 0.05$) in their expression. In excess of K^+, the expression of *D4H* (40%) and *DAT* (90%) increased. In contrast, *D4H* and *DAT* expressions decreased by 40% and 30%, respectively in K^+ deficiency.

The spray of AsA increased the expression of *D4H* and *DAT* by nearly 30% and 65%, respectively, but there were no differences between 750 or 1 500 mg/L AsA.

The dual effect of AsA and different forms of K^+ caused a significant interaction ($P \leq 0.05$) on the expression of both genes. A combination of AsA with KNO_3 increased the expression of *D4H* and *DAT* more than AsA with K_2SO_4. The highest expression of *D4H* and *DAT* (almost 3 times) happened in excess of K^+ in the form of KNO_3 and AsA while using AsA and K^+ in the form of K_2SO_4 resulted in 2.5 times more expression of *D4H* and *DAT* (Figure 2).

Peroxidase activity. There were no significant differences between forms and 15 or 30 mmol K^+, but K^+ deficiency, optimum of K^+, and excess of K^+ showed a significant difference ($P \leq 0.05$). In excess of K^+, POD activity increased by 75%, while K^+ deficiency decreased POD activity by almost 66% compared to the control plant.

The spray of 750 or 1 500 mg/L AsA increased POD activity by almost 26% and 37%, respectively.

The dual effect of AsA and K^+ caused a significant interaction ($P \leq 0.05$) on POD activity. A combination of AsA with K^+ increased POD activity by 85% (Figure 3).

Amount of H_2O_2. K^+ deficiency, optimum of K^+, and excess of K^+ showed a significant difference ($P \leq 0.05$). In excess of K^+, the H_2O_2 amount decreased almost 21%, while K^+ deficiency raised H_2O_2 content by 34%. There were no significant differences between different forms and 15 or 30 mmol K^+.

The production of H_2O_2 decreased with the application of 750 or 1 500 mg/L of AsA by almost 10% and 19%, respectively. Different forms and 15 and 30 mmol K^+ showed no significant differences.

The dual effect of AsA and K^+ caused a significant interaction ($P \leq 0.05$) on the H_2O_2 amount. A combination of AsA with K^+ reduced H_2O_2 content by 29% (Figure 3).

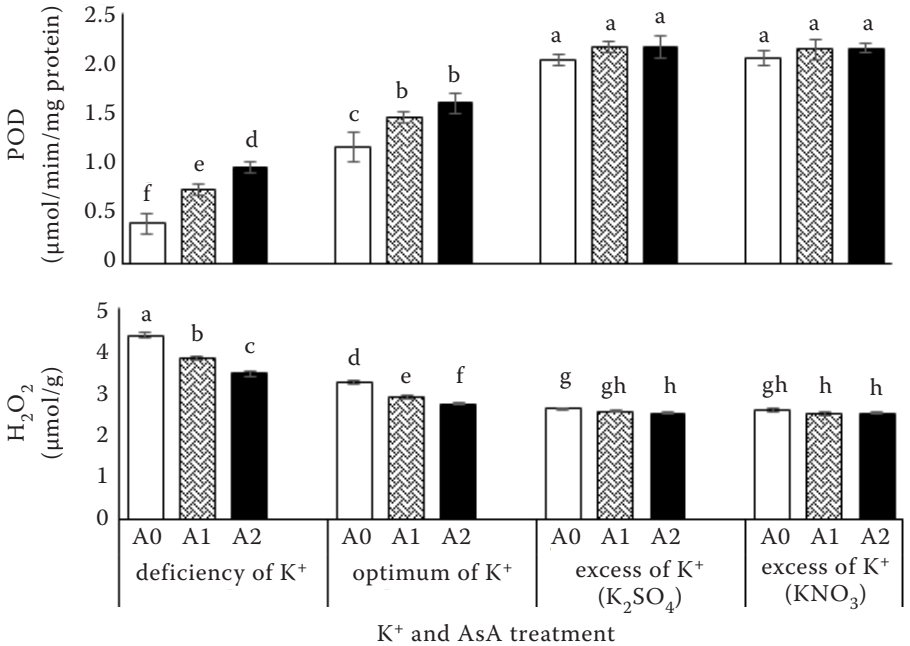

Figure 3. Peroxidase (POD) activity and H_2O_2 content in the leaves of *Catharanthus roseus* plants. Excess of potassium (K^+): 15 or 30 mmol K^+ (one column, insignificant differences between two concentration). Abbreviated letters A0, A1, and A2 used for 0 (control), 750, and 1 500 mg/L ascorbic acid, respectively. The values are the means of three independent biological replicates for each treatment. The bar lines represent the standard deviation and the different letters indicate the significant differences ($P \leq 0.05$) between different groups of plants based on Duncan's multiple range test

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DISCUSSION

There was a specific relationship between the content of vinblastine and vincristine in *C. roseus* and the downstream TIA pathway genes (*D4H* and *DAT*), the amount of tryptophan, peroxidase activity, and H_2O_2 content. Amino acids act as osmoprotective solutes to lower the osmotic potential in different plant tissues exposed to any stress. Their contributions increased in K^+ deficiency because K^+ deficiency accelerated protein degradation to form free amino acids, and the amino acids failed to synthesise the proteins and secondary metabolites successfully. Thus, free amino acids were sharply accumulated in response to stress. K^+-deficient plants usually have higher free amino acids compared to those supplied with adequate K^+. For instance, a significant increase in the free amino acid contents was reported in K^+-deficient leaves of corn (*Zea mays* L.) (Hsiao et al. 1970). Based on our results, the tryptophan content changed due to the application of K^+ treatments, inversely correlated with the amount of K^+. Also, ascorbic acid affects the content of alkaloids (Mohsen et al. 2013) and amino acids (Bassuony et al. 2008). Our results indicated that AsA acted the same as K^+ did to tryptophan content and reduced its amount as the AsA amount increased. The same results were reported for exogenous AsA on phenylalanine in *Spinacia oleracea* L. (Min et al. 2020). This change is supported by higher consumption of phenylalanine in spinach. Superior utilisation of phenylalanine supports this change in spinach, like other aromatic amino acids such as tryptophan in *C. roseus*. Tryptophan is a precursor for the biosynthesis of secondary metabolites in various plants (Dixon 2001). Decreased tryptophan can be the result of further production of secondary metabolites in AsA-treated *C. roseus*. Consequently, as tryptophan contributes to vinblastine and vincristine biosynthesis in *C. roseus*, the reduction of tryptophan caused an increase in their contents in K^+ and AsA treated plants. The reduction of K^+ decreased phenolic compounds in *Rubus fruticosus* L. (Ali et al. 2012) and scopolin in *Helianthus annuus* L. (Lehman and Rice 1972). However, there was a positive correlation between the total flavonoid and phenolic contents in *Labisia pumila* and the amount of K^+ fertilisers (Ibrahim et al. 2012). Therefore, the concentration and forms of K^+ significantly affected vinblastine and vincristine of *C. roseus* leaves, and their content increased with an excess of K^+. However, K^+ deficiency led to a decrease in them.

The uptake of K^+ by roots exhibits biphasic kinetics in response to increasing external concentrations corresponding to high- and low-affinity HAK5, AKT1, and non-selective cyclic nucleotide-gated cation channels (CNGC), which work at low and high external K^+ concentrations (Ragel et al. 2019). Under any stress conditions that directly affect K^+ acquisition, such as K^+ deprivation, high-affinity K^+ uptake systems should be transcriptionally or post-translationally activated to maintain the K^+ supply. Accordingly, all characterised HAK1-like transporters exhibiting low expression levels in roots under control conditions are highly up-regulated upon K^+ deprivation and rapidly down-regulated when K^+ is resupplied (Ragel et al. 2019). In contrast to HAK1-like transporters, KT/HAK/KUP proteins show diverse expression patterns, and most of them do not exhibit transcriptional regulation in response to K^+ deficiency (Armengaud et al. 2004). Members of the K^+-uptake carriers family have been widely associated with high-affinity K^+ uptake from the soil. However, others may function in both low-affinity or high-affinity transport and other roles related, for example, to K^+ translocation, control of water movement at the plant level, salt tolerance, osmotic/drought responses, transport of other alkali cations, and developmental processes in plants, such as root hair growth and auxin distribution. These diverse functions of KT/HAK/KUP transporters may all result from their critical roles in cellular K^+ homeostasis. KT/HAK/KUP genes are not present in animal cells, and present in all plant genomes what could indicate that they are crucial for K^+ transport in organisms facing external solutions with fluctuating and very low K^+ concentrations (Ashley et al. 2006), and this difference may reflect the importance of these transporters for the plant's way of life (Ragel et al. 2019).

Through the outward-rectifying GORK channel, K^+ efflux facilitates the fine-tuning of plasma membrane electrical potential and allows repolarisation under the circumstances such as salinity stress. For nutrient delivery to the shoots, the outward-rectifying SKOR channel releases K^+ into the xylem vessels in the root stele. In aerial tissues, an array of K^+-influx channels and KT/HAK/KUP carriers allow the uptake of the incoming K^+ into green cells. K^+ is stored inside vacuoles by NHX exchangers and released back to the cytosol by TPK and TPC1 channels, and possibly also by KT/HAK/KUP carriers at the tonoplast. The plasma membrane outward K^+ channel AKT2 releases K^+ into the phloem to return K^+ to the root

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and facilitate the uploading of photosynthates into the phloem sap (Dreyer and Uozumi 2011). Thus, the same K^+-dependent feature of the pore is employed by K^+ uptake and K^+ release channels in opposite ways-K^+ uptake channels close when external K^+ is low, K^+ release channels close when external K^+ is high (Dreyer and Uozumi 2011).

The concentrations of K^+ showed a significant effect on *D4H* and *DAT* genes expression. The final step of vinblastine and vincristine biosynthesis is regulated by *D4H* and *DAT* genes. Some evidence is available for different functions of K^+ to alter expression patterns of potassium-responsive genes in metabolic processes and transcriptional regulation. Furthermore, transcriptomic profiles of rice roots in response to K^+ (Ma et al. 2012) and *Arabidopsis thaliana* under K^+ deficiency introduce many candidate genes and regulatory pathways related to K^+ perception (Armengaud et al. 2004). Spraying AsA increased the expression of *D4H* and *DAT* genes. For instance, AsA influences the transcription levels of senescence-associated genes (Zhang 2012) and aquaporin genes in *Arabidopsis thaliana* (Qian et al. 2014). The dual effect of AsA and different forms of K^+ caused a significant interaction ($P \leq 0.05$) on the expression of both *D4H* and *DAT* genes. It looks like K^+ and AsA are involved in the expression of the related genes in the TIAs biosynthesis pathway and contribute to the production of more vinblastine and vincristine. It is demonstrated that increasing vindoline biosynthesis in *C. roseus* occurred *via* boosting and tuning the copy numbers of the enzyme's genes (Liu et al. 2021). Also, after light-treated *C. roseus*, upregulation of *D4H* and *DAT* has been observed. And the concentration of vindoline increases significantly in *C. roseus* (Vázquez-Flota et al. 2009, Liu et al. 2011). In addition, the compounds of the fungus extract result in increasing in secondary metabolite production *via* an effect on the expression of the biosynthetic pathway of vinblastine. The production of vinblastine has been increased *via* the increased activity of the biosynthetic pathway enzymes (Ishikawa et al. 2009).

K^+ contributes as a cofactor in some enzyme activities and adjusts the balance between cations and anions in different processes. It also corrects the structure of enzymes to have better efficiencies in some pathways (Prajapati and Modi 2012). AsA acts as an enzyme cofactor and precursor to produce different forms of secondary metabolites (Herrera-Martínez et al. 2013). In the TIAs pathway, d4h is an oxidoreductase enzyme, and AsA acts as an activator (www.brenda-enzymes.org) and a cofactor (enzyme.expasy.org) for this enzyme. AsA is an H^+ donor and necessary for maximal activity of d4h (www.brenda-enzymes.org). So, spraying AsA on the leave surfaces of *C. roseus* leads to more activity of d4h and increases vinblastine and vincristine contents in *C. roseus* leaves. K^+ along with AsA helps the d4h and dat enzymes activity produce higher chemical components in biosynthesis processes. Therefore, d4h and dat activities followed closely the levels of *D4H* and *DAT* expression (El-Sayed and Verpoorte 2007). And vinblastine and vincristine are produced more in the alkaloid biosynthesis pathway.

Excess of K^+ and AsA increased the POD activity. We found that the production of H_2O_2 also decreased with the application of K^+ and AsA. It suggested that POD activity decreased H_2O_2 accumulation in the leaves of *C. roseus*. The decrease of H_2O_2 due to the application of AsA is also reported in other plants like *Chenopodium quinoa* Wild. (Aziz et al. 2017). Vinblastine and vincristine dimeric compounds are built from the combination of vindoline and catharanthine monomers. POD is responsible for the combination of catharanthine and vindoline to produce α-3',4' anhydrovinblastine, which is converted to vinblastine and vincristine (Wang et al. 2016). Therefore, K^+ and AsA may increase vinblastine and vincristine biosynthesis *via* increasing POD activity.

Briefly, excess of K^+ and AsA alters several parameters affecting the vinblastine and vincristine production in *C. roseus*. K^+ and AsA led to an increased expression of related genes involved in vinblastine and vincristine biosynthesis. These genes increased the activity of the related enzymes of these pathways. Furthermore, K^+ and AsA reduce tryptophan concentration. Therefore, tryptophan contributed to the pathway to produce vinblastine and vincristine synthesis. Since vinblastine and vincristine are composed of two monomers (catharanthine and vindoline), K^+ and AsA affect peroxidase activity to provide the combination of the monomers and the vinblastine and vincristine production.

In conclusion, it seems that the increase in vinblastine and vincristine is achieved by using determined concentrations of K^+ and AsA in *C. roseus*, which is very valuable.

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