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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2023.409.418

Research Article
VTE1 and *VTE3* Gene Expression During Vitamin E Production in Sunflower (*Helianthus annuus* L.) Treated with Different Fertilization

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Abstract

Background and Objective: Sunflower is one of the important commodities in agriculture. The oil content in sunflower seeds has been widely used as cooking oil, but in Indonesia, the utilization of this oil is still relatively low. In addition, sunflowers also contain vitamin E which is useful as an antioxidant, so it can be used to reduce the risk of cardiovascular disease. This study aims to determine gene expression at the RNA level towards vitamin E biosynthesis using different fertilization treatments. **Materials and Methods:** Sunflowers that had been given different fertilizers were taken in three flowering phases, R3, R5 and R8. Flower samples were isolated until RNA was obtained. The isolation results were tested using real-time PCR to determine the relative gene expression of the *VTE1* and *VTE3* genes. After the sunflower seeds were fully ripe, vitamin E content was tested in each treatment and the results were compared with the relative gene expression obtained. **Results:** The results obtained were fluctuating, but in general, the relative gene expression obtained in the *VTE1* gene increased in the R3 phase and then decreased in the R5 and R8 phases. Whereas, in the *VTE3* gene, the relative gene expression obtained experienced an increase in the R3 and R5 phases and then decreased in the R8 phase. The highest vitamin E content was obtained by sample P3 (4218 μg mL^{-1}) and the lowest was obtained by sample P2 (1798 μg mL^{-1}). **Conclusion:** A balanced ratio of 92:46:30 kg ha^{-1} of major nutrient fertilizer involving N, P and K could increase vitamin E content in sunflowers. Such a combination exhibited stable expression of the *VTE1* and *VTE3* genes in all phases of flowering.

Key words: Sunflower, fertilization, *VTE1*, *VTE3*, gene expression

Citation: Amimy, H.H., R. Yunita, R.S. Rita, F.N. Rosadi and L. Syukriani *et al*., 2023. *VTE1* and *VTE3* gene expression during vitamin E production in sunflower (*Helianthus annuus* L.) treated with different fertilization. Pak. J. Biol. Sci., 26: 409-418.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is an annual plant originated from North America. Cultivation of this plant can be found in dozens of countries in six continents which is mostly produced as oilseed crop[1]. Apart from being used as an ornamental plant, sunflowers can also be used as industrial raw materials, such as raw materials for food, medicine and cosmetics. The potential use of sunflowers is inseparable from the content contained in these plants. One of the contents of sunflower is vitamin E[2].

Vitamin E is a compound commonly used to identify tocopherols and tocotrienols. Vitamin E is only found in organisms that can carry out photosynthesis, such as the majority of plants and some types of algae. Vitamin E in plants is useful in intracellular signaling processes, maintains cell membrane stability and improves the quality of oils and proteins. Vitamin E is also a strong antioxidant compound, so it can be used to reduce the risk of cardiovascular disease and cancer, improve immune function and prevent degenerative diseases in humans[3]. The content of tocopherol in plants of the same species can be different, one of the reasons is that the nutrients received by these plants are in the form of nutrients available in the soil or through the fertilization process.

The process of plant growth and development is strongly influenced by the nutrients received by plants. Nutrients in the soil are divided into macro and micronutrients. Macronutrients are nutrients needed by plants in large quantities, while micronutrients are the opposite. Several types of macronutrients, such as nitrogen (N), phosphor (P) and potassium (K), these three compounds are commonly used in the fertilization process[4]. Nitrogen in sunflowers plays a role in the formation of flowers and seeds, phosphor plays a role in increasing the quality of seeds and potassium plays a role in the process of growth and metabolism[5]. These main nutrients can affect sunflower metabolism, including tocopherol biosynthesis as a compound of vitamin E. This influence can be seen in the expression of genes related to tocopherol biosynthesis. Transcriptomic analysis can be used to see the effect.

Based on the tocopherol biosynthetic pathway, the *VTE1* and *VTE3* genes can be used to see the expression of tocopherol genes. This is because these genes encode key enzymes in the tocopherol biosynthetic pathway, which are tocopherol cyclase (TC) and 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase (MPBQ-MT) enzymes. The *VTE3* gene that encodes the MPBQ-MT enzyme functions to transform MPBQ compounds into 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ), while the *VTE1* gene that encodes the TC enzyme functions to transform DMPBQ into γ-tocopherol[6].

According to Alzamel *et al.*[7] the provision of nutrients in the form of fertilizers to sunflowers, both organically and inorganically, can affect growth, yield and secondary metabolites contained in the sunflower. Tocopherol as one of the secondary metabolite compounds found in sunflowers will also be affected by the provision of nutrients. The link between nutrition and tocopherol biosynthesis in several flowering phases can be identified. This study was to explore the changes in *VTE1* and *VTE3* gene expression in sunflowers treated with various fertilization treatments.

MATERIALS AND METHODS

Study area: Plant materials were grown in the field station of the Faculty of Agriculture, while transcript analysis was performed at the Biotechnological Lab of Universitas Andalas from August, 2022 to January, 2023.

Sample preparation: In total of 225 plants identified as HA1 genotypes were grown during the study. Forty-five individual plants were used as samples for each treatment as depicted in Table 1. Sample materials were collected in three phases according to Fig. 1a-c. Phase R3 is done when the flower is still in a state of bud. Phase R5 is carried out when the flower has bloomed and the disk flower begins to fill. Phase R8 is carried out when the seeds have been filled and carried out before the plants experience physiological maturity. Disk florets in the sunflower is the specific part used as a sample, this part is used because it is the place where seeds are formed in sunflowers.

Total RNA isolation: The total was extracted using Geneaid's Total RNA Mini Kit (Plant) (Geneaid, Taiwan) following the manufacturer's recommended procedures. The 100 mg of samples were crushed using liquid nitrogen and transferred into a 1.5 mL tube. Samples were added with 500 µL of RB buffer and 5 µL of β-mercaptoethanol and mixed in a vortex. The sample tube was incubated at 60°C for 5 min and subsequently, transferred to the filter column which has been connected to a 2 mL collection tube. The tube was centrifuged at 1000×g for 1 min. The filtrate was transferred into a 1.5 mL microtube and half of the total volume of the filtrate was added with absolute ethanol. The solution was homogenized and transferred into the RB column which was connected to a 2 mL collection tube. The tube was centrifuged at 1400×g for 2 min. The filtrate obtained was discarded and the RB column was added with 400 µL of WB1 solution. The tube was

Fig. 1(a-c): Flowering phase used as samples, (a) R3 phase of flowering, (b) R5 phase of flowering and (c) R8 phase of flowering

Table 1: Fertilizer composition

	Fertilizer (kg ha^{-1})		
Treatment	Nitrogen	Phosphor	Potassium
P0	16	16	16
P1	69	46	30
P2	69	23	30
P3	92	46	30
P4	92	23	30

Table 2: Primer list for real-time PCR

Gene	Primer sequence (5'-3')	Size (bp)
VTE1	F: AGCAACTACAGCATGGACCG	118
	R: TCATGCCGACTTCCCCAAAAG	
VTE3	F: ATCAGGAGATTCACCGTTGC	134
	R: TGAAACAGGGACCAGGACG	
UNK2	F: AGGAGGTAGCCGTCGTCCAGC	142
	R: AGCCTTGGAGTTCAATTGGGCCG	

centrifuged at 1400×g for 2 min. The filtrate obtained was discarded and the RB column was added with 600 µL wash buffer. The tube was centrifuged again at 1400×g for 2 min. As 50 µL of RNAse free water solution was added to the column and incubated for 2 min. The tube was centrifuged at 1400×g for 2 min. The RNA concentration and purity were determined with a Biodrop-DUO UV-Vis Spectrophotometer (Biochrom, UK).

cDNA synthesis: The RNA strand was converted into complementary DNA (cDNA) using ReverTra Ace® qPCR RT Master Mix with gDNA remover kit (Toyobo, Jepang) following the manufacturer's recommended procedures with some modifications. In total of 8.8 µL of 4× RT Master Mix solution was mixed with 1.8 µL of gDNA remover. Cocktail composition is composed of 4 µL of DNase I solution, 10 µL of total RNA and 2 µL of nuclease free water. The mixture was incubated for 5 min at 37°C. The solution was mixed with 2 µL 5× RT Master Mix II. Thermal condition in the PCR (Analytik Jena, Germany) was a cycle of 37°C for 15 min, 50°C for 5 min and 98°C for 5 min.

Primer design: The PCR oligonucleotide was designed using the primer blast tool provided at the National Center of Biotechnology Information (NCBI) developed by Ye *et al.*[8]. Primer designing was based on the *VTE1* and *VTE3* gene sequences. Primers reliability was tested for self-dimers using the Multiple Primer Analyzer[9] and double-checked using Primer3Plus software developed by Untergasser *et al.*[10] The hairpin formation was checked as suggested by Vallone and Butler[11]. The oligonucleotides were synthesized at Integrated DNA Technologies (IDT) *UNK2* gene was used as an expression gene reference (Table 2).

Real-Time Polymerase Chain Reaction (RT-PCR): *In-vitro* amplification was performed using SensiFAST™ SYBR® No-ROX kit (Bioline, UK) following the manufacturer's recommended procedures with some modifications. The composition of the cocktail used was 10 µL 2x SensiFAST™ SYBR® No-ROX kit, 1 µL of both primers (8 µM), 1 µL of cDNA and 7 µL nuclease-free water. The thermal cycle was run using the MyGo Pro (IT-IS Life Science Ltd., Ireland) condition as follows: 95°C for 1 min, 45 cycles of 95°C for 5 sec and 62°C for 30 sec (for the target

gene) or 64°C for 20 sec (for reference gene) and by a melting curve stage of 60°C for 1 min and 97°C for 1 sec. Relative gene expression was analyzed by the relative comparative Ct method, 2^{-Ct} according to Livak and Schmittgen[12] with *UNK2* as the reference gene (Table 2).

Analysis of vitamin E content: Vitamin E content was checked using a Vitamin E (VE) Colorimetric Assay kit (Elabscience, USA) following the manufacturer's recommended procedures. Sunflower seeds were weighed as much as 0.5 g and added with 4.5 mL of homogenized medium reagent in a 15 mL tube. The mixture was homogenized and centrifuged. The supernatant obtained was used as a sample. The Eppendorf tube was filled with 0.3 mL of sample and 0.6 mL of ethanol. The sample was homogenized and added with 1 mL of n-heptane, then centrifuged at 3100×g for 10 min. The supernatant was taken as much as 0.8 mL. The tube is filled with 0.1 mL of chromogenic agent and 0.05 mL of Ferrum reagent, then homogenized. Finally, the tube is filled with 0.05 mL of stop solution then homogenized and added with 1 mL of ethanol. Samples were analyzed using a spectrophotometer at a wavelength of 533 nm.

Statistical analysis: Data from real-time PCR were statistically analyzed using SPSS software version 26 with the One-way ANOVA method, Tukey's HSD Test and Pearson's correlation Test. All analysis was performed using 5% of significance level ($p<0.05$).

RESULTS AND DISCUSSION

Effect of NPK fertilizer on the vitamin E content: Based on Fig. 2, the vitamin E content differed among samples. The highest vitamin E content was obtained in sample P3 with a value of 4218 μg mL^{-1}, while the lowest content was served in sample P2 (1798 μg mL^{-1}). Compared to the P0, all samples showed lower vitamin E content, except for sample P3 which showed a higher value.

The most abundant tocopherol content in plants is α-tocopherol and γ-tocopherol. Plant seeds generally have a higher γ-tocopherol content than the α-tocopherol content, but sunflowers are unique[13]. The content of α-tocopherol in sunflower seeds is higher than the γ-tocopherol[10]. Based on Grilo *et al.*[14] the levels of vitamin E in sunflower oil which is commonly available in the market has an α-tocopherol content of 410.68 μg mL^{-1} and γ-tocopherol of 87.68 μg mL^{-1}. After being converted with the same unit of account, it showed that the levels of vitamin E in the sunflowers analyzed (Fig. 2) were greater than as reported so far. The total vitamin E content obtained has a higher value when compared to the value in the composition of the food table composition of the United States Department of Agriculture in 2020 (650.75 μg mL^{-1}).

The vitamin E content in sunflower seeds differed in each NPK fertilizer combination. The sample P3 with the highest value was treated with fertilizer and contained the highest N and P compared to the other samples. The high content of N and P in the sample seemed to influence the production of vitamin E. The P0 also has a value that is not much different from the P3 sample. This could happen because the NPK fertilizer contains balanced nitrogen, phosphor and potassium compounds and is more likely to affect the growth and vitamin E produced. The N and P have an important role in seed formation. One of the functions of N compounds in plants is seed formation, chlorophyll metabolism, protein and fat synthesis. In rice, P and K fertilization promoted early flowering, but their roles had no significant effect on the flowering duration[15].

***VTE1* and *VTE3* gene expression in sunflower:** Fertilization variation treatment affected the expression of *VTE1* and *VTE3* genes. This effect can be seen using the quantitative RT-PCR (qRT-PCR) method. According to Rao *et al.*[16] relative gene expression is usually set to 1 for the reference sample because the CT value is equal to 0, so 2° is equal to 1. Relative gene expression results with values >1 indicate increased gene expression, while values <1 indicate decreased gene expression. Figure 3 shows the *VTE1* gene data in all phases. Data on the P0 sample shows that the relative gene expression has a value close to 1 in all phases, so the P0 sample can be used as a reference sample. In phase R3 the P2 sample experienced a significant increase ($p<0.05$) compared to sample P0. There is a sample that experienced a significant decrease, which is sample P3. Other samples (P1 and P4) also experienced an increase in gene expression, but the increase was not significant. Phase R5 and phase R8 obtained similar results, both of which experienced a decrease in gene expression in all samples. After passing through phase R3, the *VTE1* gene continues to experience a decrease in gene expression.

Figure 4 analysis was performed on the *VTE3* gene. Similar to the data on the *VTE1* gene, the P0 sample on the *VTE3* gene also had relative gene expression around 1 and in all phases (Fig. 4). In phase R3, the P2 sample experienced a significant increase ($p<0.05$) compared to the control. In contrast, the P3 sample experienced a significant decrease. Samples P1 and P4 also experienced a decrease, but this decrease was not significant. Entering phase R5,

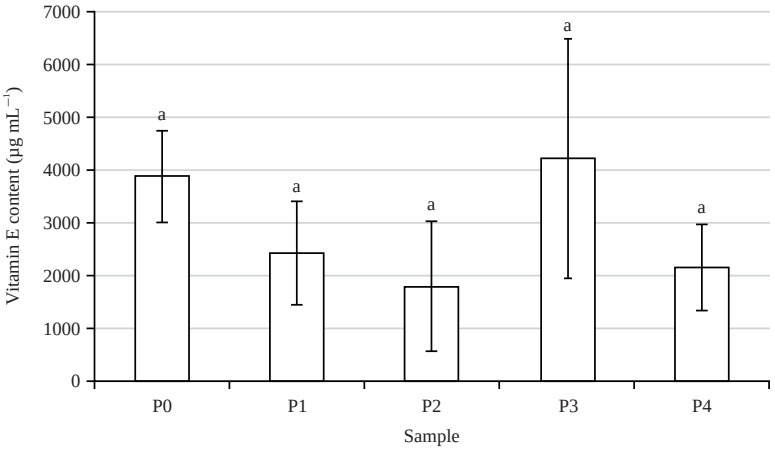

Fig. 2: Total vitamin E content in seed samples in each treatment
Data followed by the same letter are not significant at p<0.05

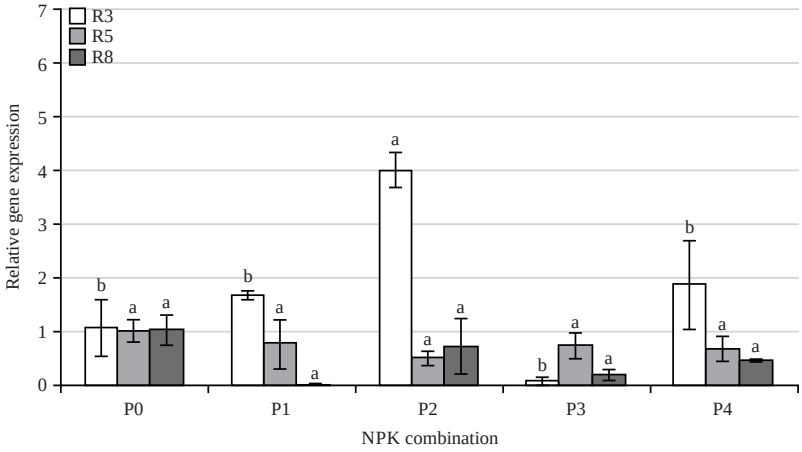

Fig. 3: Relative gene expression in *VTE1* gene of sunflower
Data followed by the same letter are not significant at p<0.05

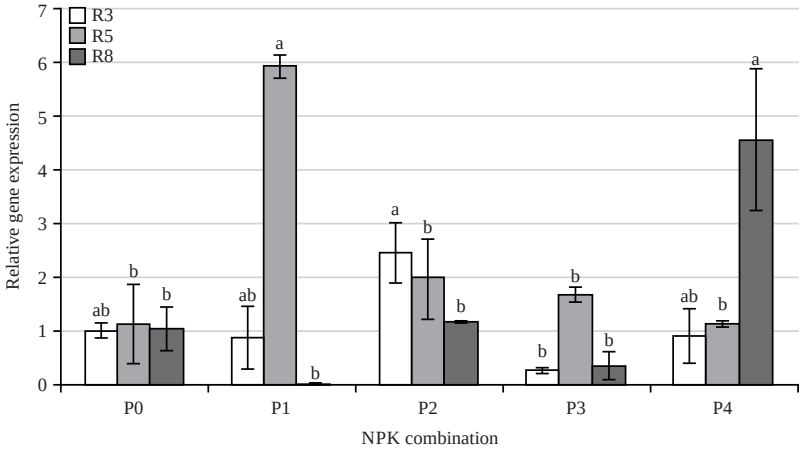

Fig. 4: Relative gene expression in *VTE3* gene of sunflower
Data followed by the same letter are not significant at p<0.05

the P1 sample had a significant increase, while the other samples also experienced an increase but not significantly. Finally, in phase R8, the P4 sample experienced a significant increase. Sample P2 has increased, while samples P1 and P3 have decreased. The P1 phase R5 sample had the highest gene expression value and the P1 phase R8 sample had the lowest value. Overall, the *VTE3* gene in each phase has a fluctuating value with a tendency to increase relative gene expression until phase R5, entering phase R8 the value of gene expression begins to decrease.

When the *VTE1* and *VTE3* gene data are compared in each phase (Fig. 5a-c), it is possible to see the difference in gene expression values between these two. In phase R3, the expression value of the *VTE1* gene was higher than that of the *VTE3* gene. Entering phase R5 and phase R8, the *VTE3* gene has a higher gene expression value than the *VTE1* gene.

Nitrogen compounds in the form of fertilizers used as treatments in experiments have a role in increasing gene expression in plants. Nitrogen plays a role in many metabolic processes, such as the metabolism of amino acids and chlorophyll. Both of these metabolic pathways have a role in the biosynthetic process of tocopherol. This increases the amount of nitrogen received by plants will also increase gene expression in the biosynthetic pathway of tocopherol[17]. Just like nitrogen, phosphorus compounds in fertilizers also have a role in increasing gene expression. Phosphorus compounds play a role in many metabolic pathways, such as photosynthesis and respiration. In addition, phosphorus is also a key macromolecule in the structure of nucleic acids and phospholipids[18]. Tocopherol biosynthesis also requires the role of phosphorus compounds, almost all the key enzymes and reactions contained in these pathways require the role of phosphorus.

The application of fertilizers with variations in composition is expected to increase the expression of the *VTE1* and *VTE3* genes in sunflowers. The results obtained on the *VTE1* gene showed a decrease in expression levels in all samples in each phase. Data in phase R3 (Fig. 5a) the majority of the sample still has a higher value than the control and the resulting relative gene expression has also increased. However, in the next phase, each sample did not get the result in the form of an increase in gene expression as expected. It is possible that the tocopherol biosynthesis process in the *VTE1* gene is inhibited. Many factors can influence this, both internal factors and external factors. One factor that allows this to occur is the influence of the weather at the time of planting. Tocopherol biosynthesis comes from the chlorophyll degradation process which is influenced by the light intensity received by plants[19]. Lack of time for plants to receive sunlight will affect this process, cloudy and rainy weather can be the cause of this. Reducing the received light intensity will inhibit chlorophyll degradation (Fig. 6), so that the formation of phytol groups which are precursors of tocopherol biosynthesis is also inhibited.

The *VTE3* gene experienced increased expression from phase R3 to phase R5. The majority of *VTE3* samples decreased when they entered phase R8. According to Sezer and Taskin[20] the *VTE1* and *VTE3* genes in plants will experience upregulation in the early stages of growth and will experience downregulation in the late maturation stage. The P4 sample in phase R8 continued to experience an increase while the other samples had decreased expression levels. This could have been due to the P4 sampling time for the analysis process which had not yet entered the late maturation stage.

However, the cause of decreased gene expression is not only due to the influence of weather, because the *VTE3* gene is not affected by these conditions. Downregulation of *VTE1* can also occur due to accumulation of carbohydrates. According to Hofius *et al.*[21] decreased gene expression in *VTE1* occurred when there was an increase in carbohydrate production tested on transgenic potatoes. The increase in carbohydrate production will be directly proportional to the increase in photosynthetic activity, one of the products of photosynthesis is carbohydrates.

These results provide an overview of the gene expression dynamic during the vitamin E biosynthesis. The *VTE1* gene experienced an increase in expression in phase R3 and finally decreased in the following phase, while the *VTE3* gene experienced an increase until phase R5 and began to decrease in phase R8. Increased gene expression in phase R3 and phase R5 could be the reason for the increased vitamin E content tested on sunflower seeds after harvest. Decreased gene expression in phase R8 does not affect the final result of total vitamin E content, because gene expression will decrease after entering the late maturation stage[16]. Vitamin E content in the P3 sample obtained the highest value, but the expression of the *VTE1* and *VTE3* genes in the sample did not experience a significant increase and decreased in several phases. The *VTE1* and *VTE3* genes in sample P3 continued to experience an increase in gene expression in phase R3 and phase R8, but the increase was not significant and tended to be smaller than in the other samples. The same thing happened to the value of the vitamin E content of the P3 sample which increased but was not significant.

Correlation of vitamin E content with *VTE1* and *VTE3* gene expression: The correlation test results will be obtained in the range of -1 to 1, if the results obtained are closer to 1, the correlation between the variables is getting closer. Based on Guilford[22] criteria, a correlation value of <0.02 is considered

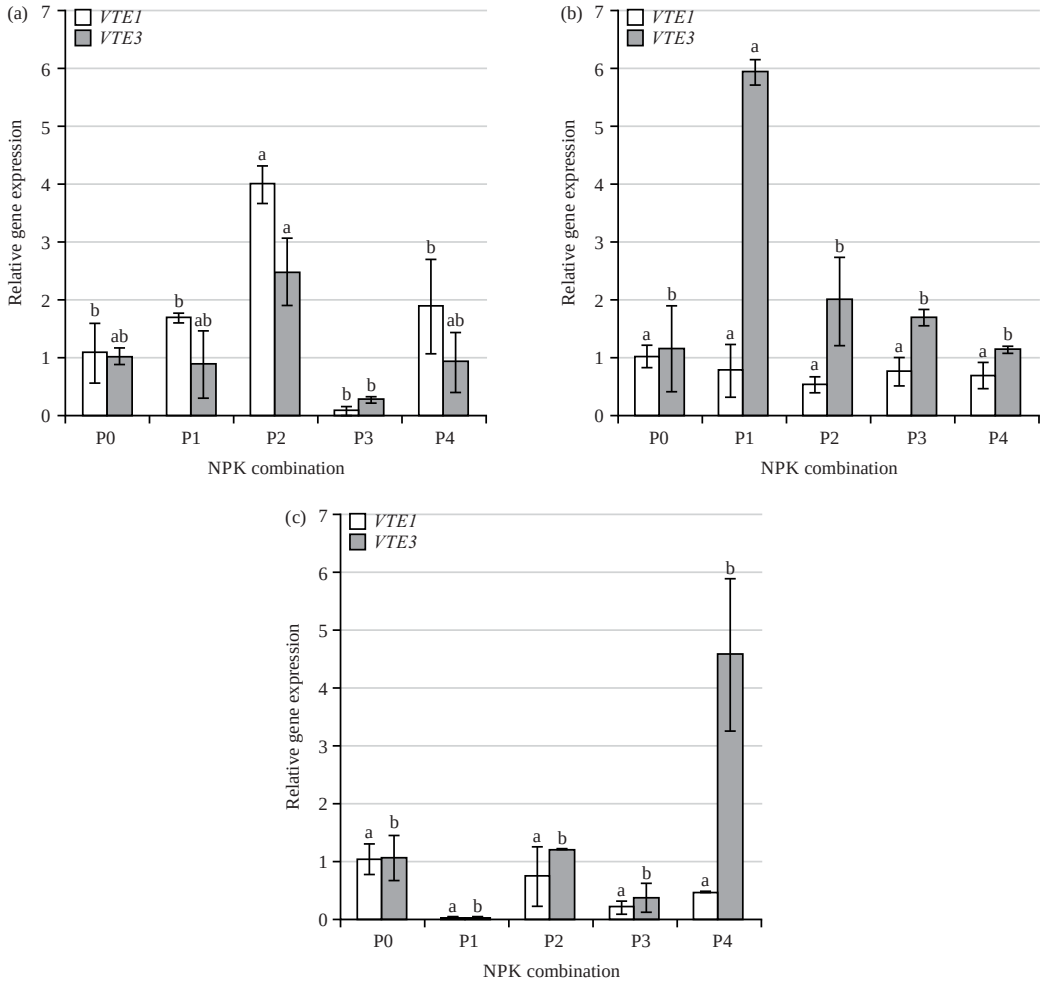

Fig. 5(a-c): Comparison of relative gene expression of *VTE1* and *VTE3* genes in each phase, (a) Relative gene expression in R3 phase, (b) Relative gene expression in R5 phase and (c) Relative gene expression in R8 phase
Data followed by the same letter are not significant at p<0.05

uncorrelated. Correlation values above this value are declared correlated. The results of the analysis can be positive and negative, indicating the direction of the correlation. A positive value indicates that the correlation between the two variables is in the same direction, while a negative value indicates an opposite direction. The results of the correlation test (Table 3) between the *VTE1* gene in phase R3 and vitamin E content were significantly correlated (p<0.05) with opposite directions. The *VTE3* gene in phase R3 was significantly correlated with the *VTE1* gene in phase R3 (p<0.01) in the same direction. Uncorrelated variables were observed between the *VTE3*gene in phase R5 and the *VTE1* gene in phase R5, the *VTE1* gene in phase R8 and the VTE1-R5 gene and the *VTE3* gene in phase R8 and the *VTE3* gene in phase R3. The correlation between *VTE1* and *VTE3* genes in all phases and vitamin E content showed that the two variables were correlated with the majority of correlations being in the opposite direction.

The correlation test results show that if vitamin E levels increase, relative gene expression will decrease and vice versa. However, theoretically, the increase in relative gene expression will be accompanied by an increase in the content of the tested compounds. This difference in results occurs because the test of vitamin E content counts all compounds detected as vitamin E, these compounds are tocopherols and tocotrienols. On the other hand, the *VTE1* and *VTE3* gene expression tests only determine the expression of tocopherol-producing genes. This is the reason why the results of the two test variables are correlated but negatively correlated.

In future research, Vitamin E content testing can be done at each phase so that the data obtained can be correlated with

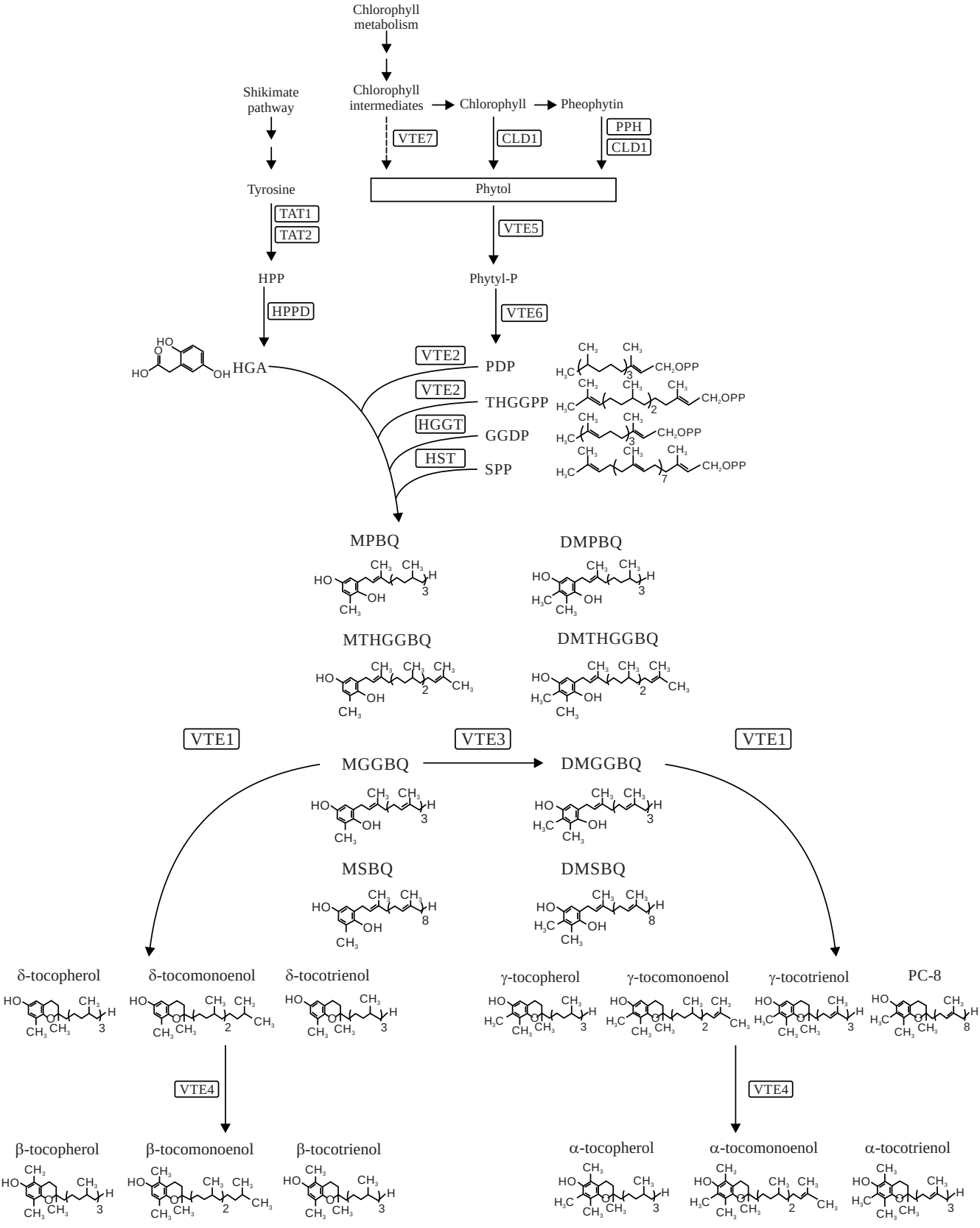

Fig. 6: Tocopherol biosynthetic pathway according to Niu *et al.*[19]

Table 3: Correlation between vitamin E content and *VTE1* and *VTE3* gene expression at each phase

	Vitamin E content	*VTE1*-R3	*VTE3*-R3	*VTE1*-R5	*VTE3*-R5	*VTE1*-R8
Vitamin E content						
VTE1-R3	-0.719*					
VTE3-R3	-0.584	0.833**				
VTE1-R5	0.364	-0.322	-0.247			
VTE3-R5	-0.261	0.066	-0.087	0.001		
VTE1-R8	-0.207	0.202	0.321	0.003	-0.550	
VTE3-R8	-0.347	0.235	-0.007	-0.025	-0.477	0.159

*Correlation is significant at the 0.05 level (2-tailed) and **Correlation is significant at the 0.01 level (2-tailed)

relative gene expression. Results that show an increase and decrease in relative gene expression based on the flowering phase can be a start for further testing using different sunflower varieties. In addition, testing on other target genes that affect vitamin E biosynthesis can be done to obtain more accurate genes to determine vitamin E content in sunflowers.

CONCLUSION

A balanced ratio of 92:46:30 kg ha^{-1} of major nutrient fertilizer involving N P and K could increase vitamin E content in sunflowers. Such a combination exhibited stable expression of the *VTE1* and *VTE3* genes in all phases of flowering.

SIGNIFICANCE STATEMENT

This research aims to elucidate the effect of different combinations of major nutrients involving nitrogen (N), phosphor (P) and potassium (K) on the expression of *VTE1* and *VTE3* genes with respect to vitamin E biosynthesis in sun flowers. The NPK combination of 92:46:30 kg ha^{-1} (P3) could increase the vitamin E content. However, expression of the *VTE1* and *VTE3* genes in the P3 treatment were down-regulated during all phases of flowering. A positive correlation between vitamin E content and gene expression is observed only with *VTE3*, while a negative correlation is observed with *VTE1*. Stable expression of both genes during all phases of flowering seemed to be an important aspect of vitamin E content improvement.

ACKNOWLEDGMENT

The authors would like to thank Universitas Andalas for funding this research through the "Hibah Guru Besar" program with contract number T/10/UN.16.17/PT.01.03/Pangan-PDU-KRP1GB-Unand/2022.

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