Indian Journal of Biochemistry & Biophysics
Vol. 61, November 2024, pp. 704-713
DOI: 10.56042/ijbb.v61i11.8591

Evaluation of phenotypic characters and total phenol content in T1 putative transgenic yellow cosmos (*Cosmos sulphureus* Cav.) with *SoSPS*1 transgene

Aziz Purwantoro[1,2]*, Tantri Swandari[1,2], Dyah Weny Respatie[2], Widhi Dyah Sawitri[2] & Rudi Hari Murti[1,2]

[1]Laboratory of Plant Breeding, Department of Agronomy; & [2]Department of Agronomy, Faculty of Agriculture,
Universitas Gadjah Mada, Yogyakarta-55281, Indonesia

Received 07 February 2024; *revised 24 July 2024*

In this study, phenotypic characters and biochemical analysis observed from leaves were carried out to evaluate the T1 population of transgenic cosmos that had inserted *SoSPS*1 (*Sucrose Phosphate Synthase* isoform in sugarcane) by floral dip using *A. tumefaciens* strain GV3101 carrying the recombinant plasmid pRI101AN-*SoSPS*1 and *npt*II gene as a selection marker. *Sucrose phosphate synthase* plays an essential role in sugar accumulation. Furthermore, the gene insertion is prospectively predicted to influence plant growth characteristics and accumulation of biochemical compounds. Information related to the impact of *SoSPS*1 overexpression on the accumulation of biochemical compounds in yellow cosmos is still relatively rare. The research material is cosmos seeds that have been transformed in previous research. Phenotypic characters according to UPOV descriptors, confirmation of the presence of the *SoSPS*1 gene, and biochemical analysis (reduction sugar, total sugar, sucrose, total phenol, and chlorophyll a, and b) were observed in the T1 plant population. The results showed the segregation of growth type, stem anthocyanin, branch density, and an increase in the character of internode length (24.64%) and diameter of the stem (12.21%). The chlorophyll content of leaves of transgenic and wild-type plants showed similar levels: increased phenol content (17.52%), reduced sugar (28.77%), and total sugar (14.71%). The higher phenol content in the transgene cosmos can make this plant a suitable resource for possible production of bioherbicide.

Keywords: Bioherbicide, Floral dip, Secondary metabolite, Sugar accumulation, Overexpression

Along with the intensification of agriculture, the use of herbicides is increasing rapidly, sometimes even not following the type and density of weeds, inappropriate growth phases, and incompatible climatic conditions. This causes the accumulation of active compounds in the soil, the abundance of certain types of weeds, accelerates the evolution of herbicide-resistant plant biotypes, and has an indirect impact on human health. These problems encourage research to find alternative bioactive compounds that can act as natural herbicides[1]. The content of bioactive compounds produced by living organisms has the potential to be used as an environmentally friendly herbicide known as bioherbicide[2]. Bioherbicides from plant secondary metabolites that have allelopathic activity with inhibitory effects on the growth of other plants are called natural herbicides[2,3].

The allelopathic activity was also shown from the flower extract of *Cosmos sulphureus* Cav. or yellow cosmos against the growth of *Cyperus rotundus* L. The content of gallic acid phenol at 14.96% of cosmos flower extract was able to inhibit growth, reduce NPK and chlorophyll content, and reduce the photosynthetic rate of *C. rotundus* weeds[4,5]. Ethanolic and methanolic extracts from *C. sulphureus* leaves and flowers present compounds such as terpenoids, phenolic, tannins, and flavonoids which have allelopathic effects[5-7]. However, studies on the potential of cosmos as a bioherbicide are still limited because generally this plant is used as an ornamental plant, refugia, and natural dye[7]. For this reason, it is necessary to increase the secondary metabolite content of gallic acid in plants so that it can optimize its role as a bioherbicide.

Efforts to increase the content of secondary metabolites in cosmos plants can be made through cultivation techniques such as optimization of SP36 fertilizer, crosses, and analysis of genetic diversity of cosmos species through RAPD and ISSR molecular markers[8,9], polyploidization using colchicine for induction of tetraploid cosmos[10], and genetic engineering of cosmos through the insertion of

---

*Correspondence:
Phone:+62 8164262446
E-mail: azizp@ugm.ac.id

*SoSPS*1 gene in planta[11]. *SoSPS*1 is a cDNA clone from sugarcane (*Saccharum officinarum*) that expresses the enzyme sucrose phosphate synthase (*SPS*)[12].

The enzyme is a key enzyme in regulating the synthesis of plant sucrose through the formation of sucrose-6-phosphate from fructose-6-phosphate and UDP-glucose. Overexpression of the *SoSPS*1 gene in sugarcane plants can increase leaf sucrose content and increase plant height and the number of clumps of transgenic plants compared to control plants[13]. Insertion of the Arabidopsis *SPS* gene in tobacco plants (*Nicotiana tabacum* cv. Xanthi) under the regulation of the 35S Cauliflower Mosaic Virus (CaMV) promoter was able to increase the concentration of sucrose in the storage organ (sink). In addition, it showed an increase in stem height, diameter, and total plant biomass weight[14]. *SoSPS*1 gene transformation in tomato plants can increase *SPS* activity 2-fold higher than in non-transgenic plants[15]. Although it is known that *SPS* gene insertion can improve phenotypic characters and sucrose content in plant organs, there is no detailed information related to phenotypic changes and accumulation of phenol compounds against *SoSPS*1 transgenes. This study is expected to provide information on the evaluation of phenotypic changes and the accumulation of phenol compounds *in SoSPS*1 transgenic plants.

Materials and Methods

The research material was yellow cosmos seeds from previous research[11]. Seeds had been transformed by floral dip with *Agrobacterium tumefaciens* strain GV3101 carrying the recombinant plasmid pRI101AN-*SoSPS*1 equipped with kanamycin antibiotic resistance selection markers. The cosmos T1 seeds used were selected from two florets (the first floret containing 11 seeds, the second floret containing 10 seeds) and 10 wild-type cosmos seeds. Cosmos plants were grown in polybags on soil and manure media (2:1). Plants are treated until the maximum vegetative phase (6-8 weeks) along with which phenotype character observations are also carried out. Molecular analysis was performed on a 3-week-old cosmos plant. Biochemical analysis was performed on the 9th and/or 10th leaf segments.

Selection of T1 yellow cosmos transgene *SoSPS*1

*SoSPS*1 transgenic T1 yellow cosmos seeds were germinated on filter paper moistened with 50 ppm kanamycin antibiotic solution for 1 week. Seeds that germinated indicated resistance to kanamycin antibiotics, so they were transplanted in polybags containing a mixture of soil and manure[11,16]. Plants were grown in a greenhouse so that phenotypic characterization and molecular analysis could be carried out for confirmation of the *SoSPS*1 transgene.

Confirmation of T1 yellow cosmos transgene *SoSPS*1

T1 yellow cosmos putative transgene *SoSPS*1 2-3 weeks after transplanting were taken 100mg of leaves to be used in DNA extraction. DNA extraction used 2% CTAB following the results of previous studies[16,17]. Extracted DNA samples were used in molecular analysis through PCR amplification. PCR analysis was used to detect the presence of *SoSPS*1 transgene in yellow cosmos plants that had been transformed by floral dip. Amplification of the *SoSPS*1 transgene used primer CaMV-35S (5′-GAAGACGTTCCAACCACG-3′) and SPS-P9 (5′ACACGGTATGCGCACAATGTA-3′).

The length of the amplified DNA bands ranged from 700-1000 bp. The PCR reaction composition consisted of 5 μL Powerpol Master Mix, 2 μL nuclease free water, 1 μL for each forward and reverse primer (10 mM), and 1 μL DNA sample. DNA amplification was performed using a PCR T100TM thermal cycler Bio-Rad with settings in the form of pre-denaturation at 95°C for 3 min followed by 39 cycles consisting of denaturation at 95°C for 30 Sec, annealing at 60°C for 30 Sec, extension at 72°C for 1 min, and final extension at 72°C for 5 min. PCR results were separated using electrophoresis (Bio-Rad) in a 1% agarose gel that has been added DNA staining and using a Smobio DNA ladder, then visualized under a UV transilluminator.

Characterization of yellow cosmos phenotype

Vegetative characteristics of yellow cosmos plants were observed when they reached their optimum vegetative phase, which occurred 6-8 weeks after transplanting. The observed characteristics include both qualitative and quantitative traits of the yellow cosmos plants. The qualitative and quantitative traits observed follow the yellow cosmos characterization guidelines published by the International Union for the Protection of New Varieties of Plants/ UPOV[18].

The qualitative characteristics of yellow cosmos investigated, including growth habitus, stem color, anthocyanin present in the stem, branch density, trichomes, leaf type, number of lobes, leaf tip form, leaf apex, upper and lower leaf color, leaf texture, and anthocyanin in leaves. Plant height, node number, internode length, stem diameter, leaf length, leaf width, and petiole are quantitative characteristics.

Biochemical observations of total phenol content

Samples of yellow cosmos plant leaves were weighed approximately 2 g and then pulverized with a blender/mortar and dissolved with distilled water. The extract obtained was then filtered in 100 mL volumetric flask and added to the full volume with the solvent used in the extraction. This solution was used as a stock solution for the analysis of total phenol content. Total phenol content analysis was measured by Folin-Ciocalteu method. A total of 1 mL of stock solution was transferred in a test tube then add 0.5 mL of Folin-Ciocalteu reagent and 1 mL of sodium carbonate and add distilled water to full volume. Then incubate for 10 min at room temperature and measure the absorbance at 750 nm. Measurements were also made of a blank solution that was prepared with all reagents but no sample solution was added. The analysis was done with 3 replicates. The results were seen based on the calibration of the gallic acid standard curve so that the content of mg gallic acid equivalents /gram sample weight was obtained[19,20].

Measurement of reducing sugar (Nelson-Somogy spectrophotometric method)

Samples of yellow cosmos leaves were weighed as much as 1 g then mashed and dissolved with distilled water to a volume of 100 mL in a volumetric flask. The solution was homogenized and filtered with filter paper then 1 mL was taken and 1 mL of Nelson C reagent was added (a mixture of Nelson A and Nelson B in a ratio of 25:1). The mixed solution was then heated in a waterbath at 100°C for 30 min, after the solution cooled down, 1 mL of arsenomolybdate reagent was added and homogenized with a vortex and then added distilled water until the volume became 10 mL. After that, the solution was homogenized and the absorbance was read using a spectrophotometer at a wavelength of 540 nm. The absorbance results were recorded and calculated based on the standard curve that had been made[21].

Total sugar measurement (Nelson-Somogy spectrophotometric method)

Leaf samples were crushed as much as 1 gram and then dissolved with 50 mL of distilled water in an erlenmeyer then added 5mL of 25% HCL and heated in a water bath at 100°C for 10 min after cooling, distilled water was added to a volume of 100 mL in a volumetric flask. The solution was homogenized and then filtered to obtain the filtrate. The filtrate was taken 1 mL of Nelson C reagent was added and heated in a 100°C water bath for 30 min. After the solution cooled, 1 mL of arsenomolybdate was added and homogenized with a vortex then added water until the volume became 10 mL. After that, the solution was homogenized and the absorbance was read using a spectrophotometer at a wavelength of 540 nm. The absorbance results were recorded and calculated based on the standard curve that had been made[21].

Sucrose measurement by spectrophotometric method

Measurement of sucrose content is done by entering the value of total sugar content and reducing sugar content into the following formula:

% sucrose = % total sugar contents - % dissolved sugar[22].

Chlorophyll a and b measurement

Determination of total chlorophyll, chlorophyll a, and chlorophyll b content in leaves was carried out using the Arnon method[23,24]. Extraction with Arnon method using 80% acetone and measurement of absorbance value of chlorophyll solution at wavelength (λ) 663 and 645 nm. Calculation of chlorophyll a, chlorophyll b, and chlorophyll total ($mg\ g^{-1}$) were calculated using the formula:

Chlorophyll a = $[12.7\ (D663)\ -2.69\ (D645)\ ]\times V1000\times W$

Chlorophyll b = $[22.9\ (D663)\ -4.68\ (D645)\ \times V\ 1000\times W$

Total chlorophyll = $[8.02\ (D663)\ -20.2\ (D645)\ \times V\ 1000\times W$

Where:

D = absorbance measured at 663/645 nm wavelength

V = final volume of chlorophyll extract in 80% acetone,

W = fresh weight of plant tissue

Data analysis

Analysis independent T-test using IBM SPSS to determine the significant difference between two treatments (T1 transgene *SoSPS*1 and wild-type plants) for each observation character.

Results

Selection of T1 yellow cosmos transgene *SoSPS*1

Transgenic plants were selected at the germination stage using 50 ppm kanamycin antibiotic (Fig. 1). In this investigation, there were 20 cosmos seeds that could germinate from both florets 1 and 2. The growing plants were molecularly validated with *SoSPS*1-specific primers. Accession A had 6 plants (A5, A6, A7, A8, A9, A10) that tested positive for the *SoSPS*1 transgene, while accession B had 8 plants

(B2, B3, B4, B5, B6, B7, B8, B10) (Fig. 2). The amplified DNA band measured 700 bp (as indicated by an arrow). Percentage of yellow cosmos plants resulting from floral dip transformation with confirmed transgenes *SoSPS*1 was 70% (Table 1).

Phenotypic characterization of T1 yellow cosmos transgene *SoSPS*1

The results showed qualitative and quantitative phenotypic character variations in yellow cosmos T1 transgenic *SoSPS*1 plants. The growth of wild-type yellow cosmos is 100% upright type, while in transgenic plants, there are variations in proportions, namely 64.29% upright type, 21.43% semi-upright type, and 14.29% spreading type. The stem color between wild-type and transgenic plants almost has the same proportion, which is dominated by green (50%). There are variations in transgenic plants, namely the category of no anthocyanins (35.71%) and weak anthocyanins (64.29%), while in wild-type plants, 100% showed no anthocyanins. Branch density in wild-type plants was only found to be characterized by moderate density (100%), while in transgenic plants there was moderate density (78.57%) and dense (21.43%). Wild-type yellow cosmos plants and *SoSPS*1 transgenic plants showed the presence of stem trichome characteristics in the same proportion (Table 2).

Fig. 1 — The selection of yellow cosmos seeds putative transgene *SoSPS*1 using kanamycin continued with the care of seedlings that passed the kanamycin selection.

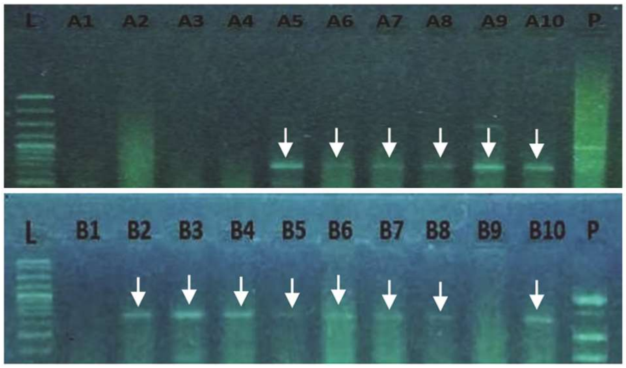

Fig. 2 — Visualization of amplification results of yellow cosmos T1 transgenic *SoSPS*1 plants. L. 1kb DNA ladder, A. Plants from florets 1 (A1-A10), B. Plants from florets 2 (B1- B10), P. plasmid pRI101 as positive control.

Table 1 — Percentage of yellow cosmos plants with confirmed transgene *SoSPS*1

Number of seeds	Number of germinated seeds	Number of positive transgene *SoSPS*1	% Positive *SoSPS*1
21	20	14	70%

Note: Percentages are based on the number of *SoSPS*1 transgenic T1 yellow cosmos seeds that germinated and grew in the greenhouse.

Wild-type and transgenic T1 yellow cosmos plants had similar proportions of leaf type (100% bipinnate board type), leaf terminal (100% multiple terminal types), and lower leaf color (100% whitish green type) characters. Two types of leaves observed in this study were pointed (42.86%) and oval (57.14%) in transgenic yellow cosmos plants, while only pointed leaf tip shapes were shown in the wild type (100%). Leaf indentation in transgenic plants showed a variation in proportion dominated by medium indentation type (71.43%), while in wild-type plants, only plants with deep indentation type were found (100%). Upper leaf color characters showed a dominance of dark green color type with a proportion above 50% in both wild-type and transgenic plants. The leaves of transgenic plants showed variations in coarse texture (57.14%) and fine texture (42.86%), while wild-type plants all showed fine texture (100%). Leaf anthocyanins in transgenic plants showed weak anthocyanins (78.57%) and moderate anthocyanins (21.43%), while wild-type plants tended to have weak anthocyanins (100%) (Table 2).

The phenotype of yellow cosmos T1 transgenic *SoSPS*1 plants in quantitative characters of stems and leaves did not show significant differences with wild-type plants, except for the characters of internode length and diameter. Transgenic plants showed an increase in internode length (24.64%) and internode diameter (12.21%). Quantitative characters of wild-type yellow cosmos plants also showed an increase but insignificant in plant height, number of internodes, leaf length, width, and petiole length (Table 3). At the seedling stage, transgenic plants showed relatively higher plant height growth than wild-type yellow cosmos plants, as well as in the vegetative growth phase, although statistically, there was no significant difference (Fig. 2). Based on this figure, the leaves of transgenic yellow cosmos plants

Table 2 — Qualitative characters of habitus, stem, and leaves of yellow cosmos transgene *SoSPS*1 and wild type plants

Character	Sub character	Proportion of sub character (%)	
		Wild Type	Transgene *SoSPS*1
A. Plants growth habitus			
1. Growth	Upright	100	64.29
	Semi upright	0	21.43
	Spreading	0	14.29
B. Trunk			
1. Stem color	Bright green	25	21.43
	Green	50	50.00
	Dark green	25	28.57
2. Anthocyanins	Absent	100	35.71
	Weak	0	64.29
	Strong	0	0.00
3. Branch density	Tenuous segments	0	0.00
	Medium	100	78.57
	Tight segments	0	21.43
4. Trichomes	Absent	100	100.00
	Present	0	0.00
C. Leaves			
1. Leaf type	Board bipinnate	100	100
	Bipinnate	0	0
2. Number of lobes	Medium	0	0
	Many	100	100
	Very many	0	0
3. Leaf tip shape	Taper	100	42.86
	Oval	0	57.14
4. Leaf apex	Shallow	0	7.14
	Medium	0	71.43
	Deep	100	21.43
5. Upper leaf color	Yellowish green	0	0
	Light green	0	21.42
	Green	33.33	21.42
	Dark green	66.67	57.14
6. Lower leaf color	Light	100	100
	Medium	0	0
	Green	0	0
7. Leaf texture	Rough	100	57.14
	Smooth	0	42.86
8. Anthocyanin	Absent	0	0
	Weak	100	78.57
	Strong	0	21.43

Table 3 — Quantitative characters of habitus (stem) and leaves of yellow cosmos transgene *SoSPS*1

	Wild type	Transgene *SoSPS*1	% increased	T-Test	P value
A. Stem					
Plant height (cm)	90.67±2.08	102.15±18.28	5,95	1.059	0.308^{ns}
Number of nodes (unit)	15.00±2.45	16.5±3.46	4,76	0.803	0.433^{ns}
Internode lenght (cm)	4.25±0.25	7.03±1.70	24,64	2.76	0.015^{*}
Diameter (mm)	9.53±0.11	12.18±2.03	12,21	2.19	0.047^{*}
B. Leaves					
Leaflenght (cm)	18.80±1.06	21.57±5.23	6.86	0.893	0.384^{ns}
Leaf width (cm)	20.79±1.86	26.35±9.00	11.79	1.041	0.313^{ns}
Petiole (cm)	9.79±0.751	10.04±1.68	1.26	0.248	0.808^{ns}

showed an oval leaf tip shape and a medium-type leaf curve. The internode length and diameter differences between wild-type and transgenic plants are also visually easy to observe. The seated appearance of the leaves from the top of the transgenic yellow cosmos plants shows a denser/fuller character, while the wild-type plants tend to be loose/not full (Fig. 3).

Biochemical observations

The chlorophyll content of the leaves including chlorophyll a, chlorophyll b and total chlorophyll showed no significant difference between the transgenic and wild type plants. This indicates that the leaves have relatively the same level of green color. The Lab* color of the leaves based on measurements using the Minolta Chromameter tool showed similar L* (brightness) values between the leaves of transgenic yellow cosmos plants and the wild type, but significantly different values of a* (green) and b* (yellow). The a* and b* values of transgenic plants were greater than those of wild-type plants, indicating that the leaves of transgenic plants have a darker green leaf color. Analysis of the biochemical content of leaf organs of yellow cosmos plant transgenic *SoSPS*1 showed an increase in the content of total phenols, reducing sugars, and total sugars in leaf organs, but caused a decrease in leaf sucrose content (Table 4).

Discussion

The result showed that 70% of yellow cosmos plants have been confirmed putative transgene *SoSPS*1 through molecular analysis (Table 1). The transgene plant percentage from this study is relatively large. Previous research on the floral dip transformation of yellow cosmos showed that the floral dip method was most efficient when the flower

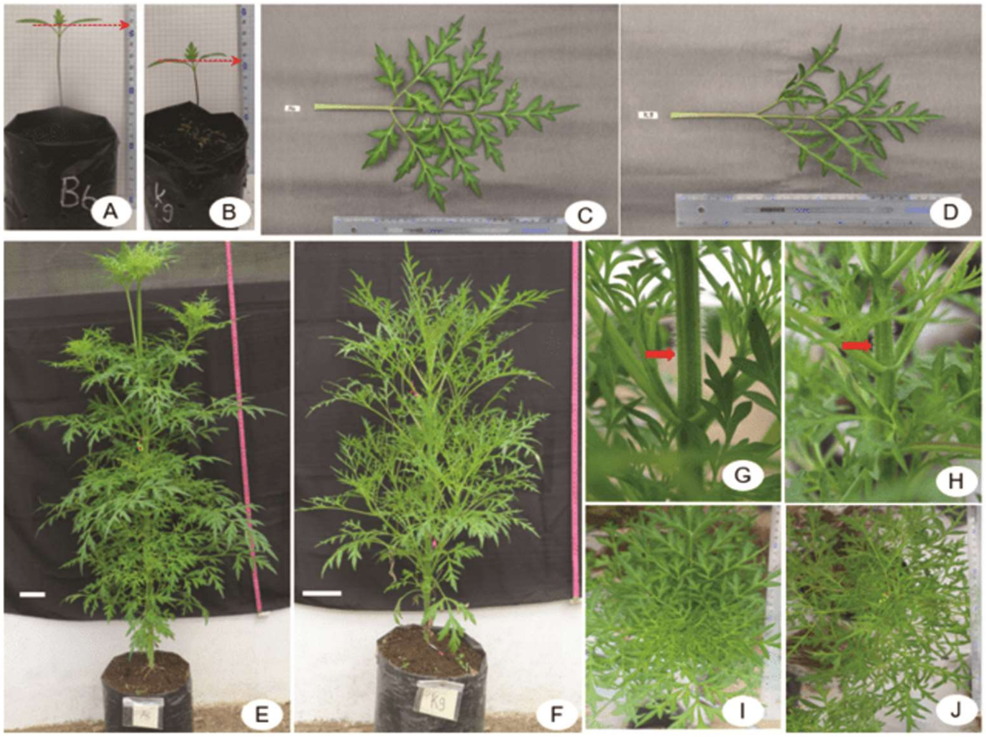

Fig 3 — Morphological characters of yellow cosmos T1 transgene *SoSPS*1 and wild-type plants. (A) T1 cosmos seedlings transgene *SoSPS*1; (B) Wild-type cosmos seedlings; (C) Leaf of cosmos T1 transgenic *SoSPS*1 plant; (D) Leaf of wildtype cosmos plant; (E) Habitus of cosmos T1 transgene *SoSPS*1 plants; (F) Habitus of wild-type cosmos plants; (G) Stem of cosmos T1 transgene *SoSPS*1 plant; (H) Stem of wild-type cosmos plant; (I) Visualization of seated leaf appearance from the top of the cosmos T1 transgene *SoSPS*1; and (J) Visualization of seated leaf view from the top of wild-type cosmos plants

Table 4 — Chlorophyll content, Lab* color, and biochemical content of yellow cosmos leaves in transgene *SoSPS*1 and wild type

	Wild type	Transgene *SoSPS*1	% increased	T-Test	P Value
A. Chlorophyll					
Chlorophyll a (mg/g)	0.226±0.039	0.232±0.024	1.31	0.438	0.668^{ns}
Chlorophyll b (mg/g)	0.412±0.069	0.421±0.045	1.08	0.422	0.678^{ns}
Total Chlorophyll (mg/g)	0.639±0.003	0.643±0.027	0.31	0.229	0.822^{ns}
B. Lab* Color					
L* Chroma	43.798±5.402	43.666±4.673	-0.15	0.067	0.947^{ns}
a Chroma	-7.204±1.352	-9.961±1.731	16.06	-4.467	0.001*
b Chroma	8.220±1.234	10.459±3.397	11.98	2.159	0.041*
C. Biochemical content					
Reduced sugar content (mg/g dry weight)	0.250±0.09	0.452±0.06	28.77	4.848	0.001*
Total sugar content (mg/g dry weight)	0.435±0.13	0.585±0.10	14.71	2.190	0.045*
Sucrose (mg/g dry weight)	0.283±0.18	0.207±0.10	-15.51	1.020	0.324^{ns}
Total Phenol (%)	0.153±0.02	0.218±0.04	17.52	2.351	0.043*

buds were dipped into *Agrobacterium* suspension. This method enhanced the transformation efficiency[11,25]. The gene encoding *neomycin phosphotransferase* II (*npt*II) transformation in yellow cosmos by floral dip has also been reported to produce 73.33% transformation efficiency based on the total number of kanamycin antibiotic-resistant plants[16]. Kanamycin was utilized as a selection marker because chimeric or albinos were excluded during selection.

The floral dip transformation method has the potential to hasten the production of transgenic plants, as it is a relatively quick way of introducing T-DNA into plants, circumventing the more tedious and labor-intensive tissue culture protocols. This method can also produce relatively uniform offspring (not experiencing chimeras) and minimize somaclonal variations that occur during the regeneration process through tissue culture; besides, this method is considered more straight forward and repeatable[26,27].

The amplification results with the primer pair CaMV-35Sand SPS-P9 produced a DNA band measuring about 700 bp (Fig 2), then the inserted *SoSPS*1 gene is a target gene that has lost the N-terminal domain (ΔSPS). If the amplified DNA band is 1kb, it is confirmed that the plant sample has an insertion of the SPS gene whose N-terminal part is still intact (full-SPS)[15]. In this study, *SoSPS*1 putative transgene T1 plants showed differences in phenotypic characteristics compared to wild-type plants including growth habitus type, stem anthocyanin, branch density, stem trichomes, leaf tip shape, leaf apex, leaf texture and anthocyanin accumulation (Table 2). The ideal transgenic plant for most research and breeding purposes would include a single, intact copy of the desired transgene inserted into a nonfunctional portion of the plant DNA without producing further DNA changes to the host plant. However, the transgene insertion site cannot be predetermined using current plant transformation techniques. This means that transgenes cannot be directed to nonfunctional regions of the genome and must be detected after insertion. T-DNA insertion can disrupt or change gene function or expression in all aspects of plant biology. Disrupting these genes may result in transgenic plants with unexpected phenotypes[28]. The observation of variation in phenotypic characters is also due to the *SoSPS*1 gene being integrated into the nucleus of yellow cosmos cells, which will then participate in the meiotic division during fertilization. The T-DNA carried by *A. tumefaciens* contains selection marker genes and desirable genes, which can be passed on to the next generation and segregated based on Mendelian Genetics[29]. Stable transformation involves incorporating of exogenous genes in the plant genome, which will lead to the expression of new traits passed on to the next generation of transgenic plants[30].

The phenotypic characters of yellow cosmos T1 transgenic *SoSPS*1 showed a significant increase in the quantitative characters of plant habitus (internode length and diameter) (Table 3). This result is because the overexpression of the *SoSPS*1 gene increases *SPS* activity and sucrose content in transgenic plants. The results of this study are the same as previous research on sugarcane (*Saccharum officinarum*)[13,31]. SPS catalyzes the conversion of fructose-6-phosphate (F-6-P) and UDP-glucose (UDP-G) to sucrose-6-phosphate (S-6-P), which serves as a substrate for sucrose phosphate phosphatase (*SPP*). The final stage involves the elimination of the phosphate group, which produces sucrose. In addition to its well-known role in sucrose biosynthesis in source leaves, it is becoming obvious that SPS has a critical role in heterotrophic cells involved in net sucrose breakdown. As a result, SPS is important in carbohydrate metabolism because it regulates the carbon partitioning between starch generation and carbohydrate (sucrose) accumulation in a variety of physiological and developmental processes[32].

The sucrose signaling pathway was used for maintaining sucrose demand, and it demonstrated the association between increasing sucrose and activation of genes regulating shoot apical meristem development (SAM). As a result, transgenic plants exhibited higher biomass and a shorter time required to convert to reproduction than those of control plants, indicating altered phyllotaxis and more rapid development through developmental stages in the transgenic plants[33]. A similar correlation was observed in clump sections, but a higher correlation was noted between *SPS* activity and sugar content. These results indicate that *SPS* overexpression, directly and indirectly, affects sugar concentration and *SAI* activity in sugarcane. In addition, *SPS* overexpression significantly increased plant height and stalk number in some transgenic lines compared to non-transgenic controls. In rice, it was shown that overexpression of the *SoSPS*1 gene significantly increased transcription and protein levels, followed by an increase in *SPS* activity and sucrose content in the leaves of transgenic lines[12,13]. Furthermore, the activity of the enzyme invertase will increase compared to the enzyme sucrose synthase, which will lead to increased growth and development of transgenic plants. This will improve the height and number of clumps in transgenic plants compared to wild-type plants[31]. The transgenic sugarcane lines overexpressing *SoSPS*1 gene had an increase in nitrogen uptake from the soil rhizosphere, thereby inducing a higher growth rate[34].

The chlorophyll a, b, and total chlorophyll content of the leaves of transgenic yellow cosmos showed no significant difference with the chlorophyll of wild-type plants. Still, based on the Lab* value, it is known that the transgenic plants have a more dominant green color than the wild type. Leaf color gives a good indication of chlorophyll content[35]. Chlorophyll is a green molecule in plant cells that plays a vital role in photosynthesis. There are two types of chlorophyll in plants, chlorophyll a and b, both of which work as photoreceptors in photosynthesis[23]. Two approaches to measure the physiological quality of leaves have been developed to monitor plant health and chlorophyll concentration: destructive and not destructive (using RGB/ Red Green Blue or Lab* color models)[24]. According to previous research[36], protein and nitrogen contents were significantly more significant in SP1 and SP3 transgenics plant strains, including potassium content in leaf and stem organs. This result may have caused the difference in leaf color. Chlorophyll can absorb sunlight and use its energy to synthesize carbohydrates from CO_2 and water[23]. Chlorophyll, a chemical that captures light during photosynthesis, increases in the presence of glucose. In rice seedlings growing in the dark, glucose accumulation enhances chlorophyll synthesis, but as glucose concentration rises, the effect on photosynthesis becomes suppressive. Very high glucose concentrations have been proven to inhibit chloroplast development. This reduction could be attributed to inhibited transcription of photosynthetic genes controlled by glucose and sucrose molecules[37]. Thus, overexpression of *SoSPS*1 allows for glucose accumulation alterations, affecting leaf chlorophyll development.

Biochemical analysis of the leaves of yellow cosmos T1 transgenic *SoSPS*1 plants showed an increase in reducing sugars, total sugars, and total phenol content except sucrose content (Table 4). These results are different from previous research, in which the presence of *SPS* transgenes in several transgenic plant strains can be expressed at the translational level and increase leaf *SPS* activity compared to wild-type sugarcane plants, resulting in an increase in sucrose content in leaf organs[38,39]. Increased *SPS* activity led to a reduction in starch and an increase in sucrose in tomato leaves but no increase in sucrose content in the fruit. These results indicate that *SPS* is involved in the regulation of carbon partitioning[40].

Since the discovery of sucrose biosynthesis, much has been learned about the control and significance of sucrose in plant functional biology. Sucrose is the primary result of photosynthesis in higher plants, as well as a transport molecule in growth, development, storage, signal transduction, and stress response[41]. This study revealed that leaf sucrose accumulation was lower than in wild-type plants. This result assumes that yellow cosmos plants require carbohydrates in simple forms, specifically glucose and fructose, to maintain their growth during the vegetative period. SuSy and INV are enzymes that help decompose sucrose[42]. The total phenols in yellow cosmos leaves with the *SoSPS*1 transgene increased compared to wild-type plants. These findings are consistent with previous research suggesting that the accumulation of carbon molecules (glucose, fructose, mannitol, maltose, and sucrose) influences the accumulation of secondary metabolites and the expression of several essential genes. The content of soluble sugars in plants, particularly leaf organs, can trigger the accumulation of secondary metabolites, particularly total phenols content (gallic acid)[43-45]. The accumulation of gallic acid in yellow cosmos extract significantly reduced the number of mother shoots, daughter shoots, and roots, as well as the length of mother leaves, rhizome length, roots, and total leaf area of the weed *C. rotundus*. These findings suggest that cosmos extract has the potential for controlling *C. rotundus*[4,5].

Conclusion

The yellow cosmos plants transgene *SoSPS*1 caused an increase in plant phenotypic characteristics, especially the length and diameter of stem internodes. Destructive and non-destructive leaf color characters showed similarities between the leaves of transgenic plants and the wild type. Biochemical analysis of leaves showed increased phenol, reducing sugar, and total sugar content.

Acknowledgment

This study was supported by the Academic Excellence 2023 scheme (7725/UN1.P.II/Dit-Lit/PT.01.03/2023) from Indonesian Ministry of Education, Culture, Research and Technology. The binary vector, primer set, and *Agrobacterium tumefaciens* strains were provided by Prof. Bambang Sugiharto at Center of Development of Advanced Science and Technology (CDAST), University of Jember, Indonesia. We also thank to Laboratory of Plant Breeding, Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada for providing research facilities.

Conflict of interest

All authors declare no conflict of interest.

References

1 Hasan M, Ahmad-Hamdani MS, Rosli AM & Hamdan H, Bioherbicides: An Eco-Friendly Tool for Sustainable Weed Management. *Plants*, 10 (2021) 1212.

2 Roberts J, Florentine S, Fernando WGD & Tennakoon KU, Achievements, Developments and Future Challenges in the Field of Bioherbicides for Weed Control: A Global Review. *Plants*, 11 (2022) 2242.

3 Marques MEM, De Carvalho AC, Yendo ACA, Magedans YVS, Zachert E & Fett-Neto AG, Phytotoxicity of *Quillajal ancifolia* Leaf Saponins and Their Bioherbicide Potential. *Plants*, 12 (2023) 663.

4 Respatie DW, Yudono P, Purwantoro A & Trisyono YA, Effect Spraying Volume of *Cosmos sulphureus* Cav. Flower Extract on Weed Dominance and Soybean Yield. *IOP Conf Ser: Earth Environ Sci*, 662 (2021) 012017.

5 Respatie DW, Yudono P, Purwantoro A & Trisyono YA, The potential of *Cosmos sulphureus* flower extract as a bioherbicide for *Cyperus rotundus*. *Biodiversitas*, 20 (2019) 3568.

6 Andrushchenko O, Vergun O & Rakhmetov D, Antioxidant capacity of *Cosmos sulphureus* plants grown in the temperate climate. *Plant Introd*, 93-94 (2022) 37.

7 Ghayal N, Biware M & Gharpure P, Phytotoxic Effects of Leaf Leachates of Invasive Weeds *Cosmos sulphureus* and *Xanthium strumarium* on Agricultural Crops. *Biosci Biotech Res Asia*, 15 (2018) 821.

8 Oku T, Takahashi H, Yagi F, Nakamura I & Mii M, Hybridisation between chocolate cosmos and yellow cosmos confirmed by phylogenetic analysis using plastid subtype identity (PSID) sequences. *J Hortic Sci Biotech*, 83 (2008) 323.

9 Rodríguez-Bernal A, Piña-Escutia JL, Vázquez-García LM & Arzate-Fernández AM, Genetic diversity of Cosmos species revealed by RAPD and ISSR markers. *Genet Mol Res*, 12 (2013) 6257.

10 Verma RC, Dass P, Shaikh N & Khah MA, Cytogenetic investigations in colchicine induced tetraploid of *Cosmos sulphureus* (Asteraceae). *Chromosome Botany*, 12 (2017) 41.

11 Purwantoro A, Irsyadi MB, Sawitri WD, Fatumi NC & Fajrina SN, Efficient floral dip transformation method using *Agrobacterium tumefaciens* on *Cosmos sulphureus* Cav. *Saudi Journal of Biological Sciences*, 30 (2023) 103702.

12 Bilska-Kos A, Mytych J, Suski S, Magoń J, Ochodzki P & Zebrowski J, Sucrose phosphate synthase (SPS), sucrose synthase (SUS) and their products in the leaves of *Miscanthus* × *giganteus* and *Zea mays* at low temperature. *Planta*, 252 (2020) 23.

13 Anur RM, Mufithah N, Sawitri WD, Sakakibara H & Sugiharto B, Overexpression of Sucrose Phosphate Synthase Enhanced Sucrose Content and Biomass Production in Transgenic Sugarcane. *Plants*, 9 (2020) 200.

14 Park JY, Canam T, Kang KY, Ellis DD & Mansfield SD, Over-expression of an arabidopsis family A sucrose phosphate synthase (SPS) gene alters plant growth and fibre development. *Transgenic Res*, 17 (2008) 181.

15 Fabriani S, Agustien I, Sawitri WD & Sugiharto B, Genetic Transformation and Expression of Sucrose Phosphate Synthase Mutant in Tomato Plant. *Bioteknol Biosains Indones,* 6 (2019) 130.

16 Irsyadi MB, Sawitri WD & Purwantoro A, Molecular and phenotypic characteristics of T1 transgenic yellow cosmos (*Cosmos sulphureus*) carrying neomycin phosphotransferase II gene. *Biodiversitas*, 23 (2022) 12456.

17 Irsyadi MB, Kartika Sari S, Oktiastuti E & Rineksane IA, Rapid genomic DNA extraction for Soybean (*Glycine max* L. Merr) using modified CTAB protocol to obtain high-quality DNA. *Indian J Biochem Biophys*, 61 (2024) 97.

18 UPOV, International Union For The Protection of New Varieties of Plants : Cosmos Cav. Technical Working Party for Ornamental Plants and Forest Trees at its forty-fifth session; UPOV (2012).

19 Ewané CA, Lepoivre P & Lassois L, Involvement of phenolic compounds in the susceptibility of bananas to crown rot.A review. *Biotechnol Agron Soc Environ*, 16 (2012) 393.

20 Liu XY, Ou H, Gregersen H & Zuo J, Deep eutectic solvent-based ultrasound-assisted extraction of polyphenols from *Cosmos sulphureus*. *J Appl Res Med Aromat Plants*, 32 (2023) 100444.

21 Shao Y & Lin AHM, Improvement in the quantification of reducing sugars by miniaturizing the Somogyi-Nelson assay using a microtiter plate. *Food Chem*, 240 (2018) 898.

22 Borji A, Borji Fz & Jourani A, A New Method for the Determination of Sucrose Concentration in a Pure and Impure System: Spectrophotometric Method. *Int J Anal Chem*, (2017) 1.

23 Khaleghi E, Arzani K, Moallemi N & Barzegar M, Evaluation of Chlorophyll Content and Chlorophyll Fluorescence Parameters and Relationships between Chlorophyll a, b and Chlorophyll Content Index under Water Stress in Olea europaea cv. *World Academy of Science, Engineering and Technology (WASET)*, 62 (2012) 1154.

24 Ali MM, Al-Ani A, Eamus D & Tan DKY, A New Image Processing Based Technique to Determine Chlorophyll in Plants. *Am-Eur J Agric Environ Sci*, 12 (2012) 1323.

25 Muchyiddin MGAA, Sawitri WD, Irsyadi MB, Swandari T & Purwantoro A, Segregation analysis and floral phenotype of T2 transgenic yellow cosmos (*Cosmos sulphureus* Cav.) carrying neomycinphosphotransferase II gene in second generation. *Biodiversitas*, 25 (2024) 1711.

26 Davis AM, Hall A, Millar AJ, Darrah C & Davis SJ, Protocol: Streamlined sub-protocols for floral-dip transformation and selection of transformants in *Arabidopsis thaliana*. *Plant Methods*, 5 (2009) 3.

27 Coomber SA & Feldmann KA, Gene Tagging in Transgenic Plants. In: *Transgenic Plants: Engineering and Utilization*, (Ed. by Kung Shain-dow, Wu Ray; Academic Press Inc, California, 1993, 181.

28 Wilson AK, Latham JR & Steinbrecher RA, Transformation-induced Mutations in Transgenic Plants: Analysis and Biosafety Implications. *Biotechnol Genet Eng Rev*, 23 (2006) 209.

29 Anami S, Njuguna E, Coussens G, Aesaert S & Van Lijsebettens M, Higher plant transformation: principles and molecular tools. *Int J Dev Biol*, 57 (2013) 483.

30 Abiri R & Valdiani, A Critical Review of the Concept of Transgenic Plants: Insights into Pharmaceutical Biotechnology and Molecular Farming. *Curr Issues Mol Biol*, 18 (2016) 21.

31 Mulyatama RA, Neliana IR, Sawitri WD, Sakakibara H, Kim KM & Sugiharto B, Increasing the Activity of Sugarcane Sucrose Phosphate Synthase Enhanced Growth and Grain Yields in Transgenic Indica Rice. *Agronomy*, 12 (2022) 2949.

32 Ma P, Zhang X, Chen L, Zhao Q, Zhang Q, Hua X, Wang Z, Tang H, Yu Q, Zhang M, Ming R & Zhang J, Comparative analysis of sucrose phosphate synthase (*SPS*) gene family between *Saccharum officinarum* and *Saccharum spontaneum*. *BMC Plant Biol*, 20 (2020) 422.

33 Nguyen QA, Luan S, Wi SG, Bae H, Lee DS & Bae HJ, Pronounced Phenotypic Changes in Transgenic Tobacco Plants Overexpressing Sucrose Synthase May Reveal a Novel Sugar Signaling Pathway. *Front Plant Sci*, (2015) 1216.

34 Wulandari FE, Suherman, Handoyo T, Kim KM & Sugiharto B, Impact of transgenic sugarcane overexpressing *SoSPS*1 gene on bacterial diversity, enzyme activity and minerals content in soil rhizosphere. *J Crop Sci Biotechnol*, 24 (2021) 61.

35 Ali M, Abbasi BH, Ahmad N, Ali SS, Ali S & Ali GS, Sucrose-enhanced biosynthesis of medicinally important antioxidant secondary metabolites in cell suspension cultures of *Artemisia absinthium* L. *Bioprocess Biosyst Eng*, 39 (2016) 1945.

36 Nur Sudraj AB & Sugiharto B, Comparative Evaluation of Nutritional and Mineral Composition Between Transgenic Sugarcane Overexpressing *SoSPS1* Gene and Non-transgenic Counterpart. *Pak J Biol Sci*, 23 (2020) 1424.

37 Siddiqui H, Sami F & Hayat S, Glucose: Sweet or bitter effects in plants-a review on current and future perspective. *Carbohydr Res*, 487 (2020) 107884.

38 Budeguer F, Enrique R, Perera MF, Racedo J, Castagnaro AP, Noguera AS & Welin B, Genetic Transformation of Sugarcane, Current Status and Future Prospects. *Front Plant Sci*, 12 (2021) 768609.

39 Miswar M, Sugiharto B, Soedarsono J, Moeljapawiro S, Transformation of Sucrose Phosphate Synthase Gene (*SoSPS1*) using Agrobacterium tumefaciens to Improve Sugar Synthesis In Sugarcane (*Saccharum officinarum* L.). *Berkala Penelitian Hayati,* 12 (2007) 137.

40 Worrell AC, Bruneau JM, Summerfelt K, Boersig M & Voelker TA, Expression of a Maize Sucrose Phosphate Synthase in Tomato Alters Leaf Carbohydrate Partitioning. *Plant Cell*, 3 (2019) 1121.

41 Salerno G, Origin of sucrose metabolism in higher plants: when, how and why? *Trends Plant Sci*, 8 (2003) 63.

42 Li W, Huang D, Wang B, Hou X, Zhang R, Yan M & Liao W, Changes of starch and sucrose content and related gene expression during the growth and development of Lanzhou lily bulb. *PLoS One*, 17 (2022) 12.

43 Singh J, Sabir F, Sangwan RS, Narnoliya LK, Saxena S & Sangwan NS, Enhanced secondary metabolite production and pathway gene expression by leaf explants-induced direct root morphotypes are regulated by combination of growth regulators and culture conditions in *Centella asiatica* (L.) urban. *Plant Growth Regul*, 75 (2015) 55.

44 Osman NI, Jaafar Sidik N & Awal A, Efficient enhancement of gallic acid accumulation in cell suspension cultures of *Barringtonia racemosa* L. by elicitation. *Plant Cell Tiss Organ Cult*, 135 (2018) 203.

45 Ramakrishnan G, Razack SA, Ravi L & Sahadevan R, Fabrication of phyco-functionalized zinc oxide nanoparticles and their *in vitro* evaluation against bacteria and cancer cell line. *Indian J Biochem Biophys,* 60 (2023) 770.