Chapter 5

Agrophysiological and Biochemical Characterization

5.1 Introduction

Genetic transformation is commonly used for plant improvement and fundamental research. It offers an important prospect in mounting crop production and diversification of the nutritional base. Methodologies for the generation of transgenic crops are frequently being improved and crops containing multiple transgenes are widely cultivated [380]. Transformation may or may not result in the incorporation of a variable number of transgene copies at different locations in the plant genome. One aspect of the procedure that cannot be controlled presently is the precise location of the transgene insertion within the host genome which may have implications for transgene stability and potential for unintended effects on overall plant metabolism by the formation of either new metabolites or altered levels of existing metabolites [130,131]. Therefore, risk of inadvertent effects due to transgene integration is one of the major concerns. Moreover, several studies often focus on the risk that a transgene may not show the desired phenotypic effect if the transformants are moved from *in vitro* to either the controlled greenhouse environment or to the more variable field conditions. However, few studies have reported potentially unintended phenotypic effects of transgenes in transgenic crops exposed to a variety of agroecological conditions [381]. Moreover, different cultivars of the same crop adapt very differently in response to environmental cues at different growth stages, even though they are accustomed to precisely the same environment. These altogether may invariably affect the nutritional status of closely related genotypes of a species [382, 383]. It is distinguished from evolutionary and ecological studies on wild plants that genotype × environment interactions (G x E) can have a greater influence, suggesting that analogous interactions might occur in GM plants exposed to diverse agroecological conditions, including greenhouse versus field environments [384]. These interactions are, to a great extent, influenced by the differential expression of gene(s) and metabolite acquisition [61, 385]. Development of globally cultivable elite cultivars or transgenic lines to overcome these limitations is the major challenge for the researchers working on sweet potato. Therefore, it is pertinent to analyze transgene mediated metabolic sift(s) in the transgenic plants. This further necessitates the development of high throughput approaches to identify the secondary effects of genetic modifications. Different approaches may be applied in order to categorize the potential secondary effects and a comprehensive investigation of the level of primary and secondary metabolites is one of the best possible ways to assess the effect(s) in comparison to the wild types grown in identical conditions. In addition,

morphological abnormalities and/or differences in transgenic crops are one of the major concerns in case of the development and adoption of transgenic crops. Since, in case of sweet potato itself the first successful transformation protocol leading to the production of sweet potato transformants by *A. rhizogenes* presented morphological abnormalities [250].

In order to increase the nutritional status of sweet potato in terms of protein, AmA1 was overexpressed. The AmA1 protein is rich in most essential amino acids and therefore, it can be anticipated that its overexpression in protein deficient crops like sweet potato can increase the overall protein content. Transgene mediated proteome rebalancing is already discussed in Chapter 4 (Section 4.4). Since enzymes involved in the metabolic pathways are also proteins, consequently, a metabolic shift may occur. One of the objectives of this study was to examine the extent of change(s) in metabolites as well as the agrophysiological aspects of transgenic events. Secondary metabolites will be discussed in next Chapter. This Chapter deals with the primary metabolites as well as a comparative agrophysiological characterization of the transgenic lines *vis-à-vis* to the wild types. To negate the possibility of natural variations and/or somaclonal variations amongst different transgenic lines another wild type variety was also considered besides cv. SP-6. The transgenic lines were raised in the genetic background of cv. SP-6 which is an orange-fleshed sweet potato (OFSP) variety and a close white-fleshed relative (WFSP) was also included in this study. The varieties henceforth are designated as OFSP-6 and WFSP-WT, respectively.

5.2 Materials and methods

5.2.1 Plant growth and maintenance

The transgenic and wild types were grown in parallel in the greenhouse in identical conditions as described in **Section 4.2.1**.

5.2.2 Assessment of agrophysiological traits

The leaf area of the 3-5th leaf from the shoot apex was measured as described earlier and calculated using LeafJ with ImageJ software (http://rsb.info.nih.gov/ij/) [386]. Rate of photosynthesis was quantified with a portable photosynthesis measurement system (GFS3000; Waltz). The photosynthetic potential was determined on the basis of single leaf measurements of

5-7 leaves from each plant and was evaluated after 8-10 weeks of plantation under standard atmospheric (360 ppm CO_2) and light conditions (750 μmol $m^{-2}s^{-1}$). A fully expanded second or third leaf from the shoot apex was held in the chamber for 2-3 min until the rate of photosynthesis was in a steady-state condition. Identical sized mature tubers were collected from both cultivars and the average weight and diameter were measured.

5.2.3 Evaluation of tuber color difference

The color of tubers was determined colorometrically using high-performance color measurement spectrophotometer (HunterLab Ultrascan Vis) coupled with EasyMatch QC software. The measurements were performed over 360-780 nm wavelengths, as per manufacturer's recommendation.

5.2.4 Biochemical and proximate analysis

For proximate analyses, tubers of both the cultivars were washed, peeled individually, sliced into smaller pieces, and oven-dried at 40ºC for 18 h. Furthermore, each sample was powdered using a laboratory scale grinder. Total moisture, ash and crude fiber were determined in triplicates according to the standard method of the Association of Official Analytical Chemists [387]. Moisture content was determined gravimetrically by the weight loss after drying the samples in a hot-air oven until constant weight was achieved. Total ash content was measured after igniting the samples in a muffle furnace at 550-600ºC to acquire a constant weight. Crude fibers were determined according to the standard AOAC method. In brief, 2 g of dried samples were boiled in 1.25% H_2SO_4 for 30 min with bumping chips. The samples were then filtered through gooch crucible and the residue obtained was subsequently washed with the boiling water repeatedly to eliminate the acid content. Residue was again boiled with 1.25% NaOH for 30 min. It was then filtered and washed with water and dried. The dried sample was placed in a crucible and ignited in the furnace at 600 ± 15°C for 2-3 h, then cooled. The weight of the residue was recorded and the percent crude fiber content was calculated via the loss in weight on and before ignition. The total protein was quantified by micro-Kjeldahl method and protein was calculated from nitrogen content multiplied by a factor of 6.25 [387].

To carry out biochemical analyses, tubers were lyophilized and grounded into fine powders. Total carbohydrate content in both the cultivars was determined by Anthrone method,

using standard protocol with few modifications using D-glucose as standard. Further reducing sugar was determined by Nelson-Somogyi method [388].

5.2.5 Assessment of amino acid content

Total free amino acids were quantified by ninhydrin method as described earlier with few modifications [388]. In addition, amino acids were quantified by GC–MS analysis as described earlier [389]. Briefly, 100 mg of tuber representing at least four biological replicates was homogenized in 1.4 ml of 100% methanol with 50 µl of ribitol as internal standard (2 mg ml^{-1}) and extracted for 15 min at 70°C. The extract was mixed with 1 volume of water and centrifuged at 2200 x *g*. Subsequently, the methanol/water supernatant was aliquoted and dried *in vacuo* for 9–16 h. The dried residue was resuspended and derivatized using 80 µl of 20 mg ml^{-1} methoxyamine hydrochloride in pyridine for 90 min at 30°C followed by a 30 min treatment with 80 µl of MSTFA at 37°C. Furthermore, 40 µl of retention time standard mixture was added prior to trimethylsilylation. The derivatized extracts were diluted 10-fold in *n*-heptane, and a sample volume of 1 µl was injected in splitless mode into the analyzer (Shimadzu GCMS-QP 2010 plus). The mass spectrometer was tuned according to the manufacturer's recommendations. GC was performed on an Rtx5MS-30 m column with 0.25 mm ID and df 0.25 (Restek). The injection temperature was set at 260°C, interface was set at 270°C, and ion source was adjusted to 230°C. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The analysis was performed using the temperature program [389]. Mass spectra were recorded at 2 scan $s^{-1}$with an *m*/*z* 40–600 scanning range. Peaks were assigned and quantified, and all data were normalized to the mean response calculated for the wild-type control of each replicate, to allow comparison between the samples. Individual wild-type values were normalized in the same way as per [390]. The recovery of small representative amounts of each metabolite through the extraction, derivatization, storage, and quantification procedures has been followed and documented as detailed previously [389]. Targeted compounds were analyzed and identified by comparing their retention times and mass spectra with those in the NIST or Wiley library.

5.2.6 Water holding capacity (WHC)

WHC was determined using a modified method of [391]. Briefly, 1 g of lyophilized tissue in triplicate was hydrated in 30 ml Milli-Q water containing 0.02% azide as a bacteriostat.

Samples were centrifuged at 3,000 x *g* for 20 min at room temperature after equilibrating it for 18 h. The supernatant was removed carefully and residual water was drained out. Sample fresh weight was recorded before drying. WHC was calculated as the amount of water retained by the pellet (g/g dry weight) after residual dry weight.

5.2.7 Evaluation of storage performance

The tubers were harvested from eight different plants (two from each experimental plot). To evaluate degradation of starch and cellulose during storage, harvested tubers were stored at room temperature and measured at every 15-d interval. Total starch content was determined by Anthrone method with few modifications [388]. Glucose content in the samples was extrapolated using D-glucose as standard and the starch content was determined by multiplying it with a factor of 0.9. Total cellulose content was determined by modifying the method of acidolysis with acetic/nitric reagent followed by the treatment with H_2SO_4. Acid hydrolyzed cellulose was then quantified by anthrone method using standard procedures as described by **Sadasivam and Manikam** (**1992**) [388].

5.2.8 Structural and chemical characterization

For structural analysis of composite samples, SEM was conducted using a scanning electron microscope (Model 6390 LV; JOEL Co., Ltd.). The dried lyophilized samples were coated with 10-15 nm thickness of platinum using a JEOL 1600 Auto Fine Coater. The samples were examined under the SEM with an accelerating voltage of 10-15 KV.

The Fourier transform infrared spectroscopy (FT-IR) spectra were analyzed to determine the different functional groups present in the compounds, which enabled partial identification of the compounds. The compounds were first mixed with about 5-7 mg of potassium bromide (KBr) to obtain a fine mixture. This was then taken in a sample holder and then placed under a hydraulic press. The hydraulic press delivers 10 ton pressure to produce a KBr compound tablet which was then placed in the sample holder for subsequent characterization. The X-ray diffraction (XRD) pattern of the sample were carried out with CuKα radiation using (λ = 1.54056 Å) Rigaku XRD Instrument. The powdered sample was mounted on the sample holder for characterization. The scan speed for characterization was 0.5 °/ min.

5.2.9 Statistical analysis

Statistical significance of the data was analysed by the unpaired student's *t*-test method using Graphpad prism 5 software. $P < 0.05$ was considered to be statistically significant and the results were expressed as mean ± SE.

5.3 Results

5.3.1 Assessment of the agrophysiological traits

The transgenic events were grown in parallel with the wild types under the same experimental conditions to investigate the extent of miscellany between them. Apparently no morphological differences were found between the OFSP-6 and the transgenic events. However, genetic differences between OFSP-6 and WFSP-WT were evident at morphological level (**Fig. 5.1 A-D**). Additionally, the average yield was significantly higher ($p < 0.05$) in most of the transgenic events (**Fig. 5.2 A**). Nonetheless, average weight and diameter were not different significantly (**Fig. 5.2 B and C**). Contrastingly, the average number of tubers per plant was increased significantly in transgenic events that might have possibly contributed more towards a greater harvest index (**Fig. 5.2 D**). The average yield and weight of both the wild types tubers were quite distinct. Average weight and diameter of the tubers of OFSP-6 were found to be more than 2- and 4-fold higher when compared with WFSP-WT. Moreover, the average yield was about 2-fold higher in OFSP-6 than that of WFSP-WT (**Fig. 5.2 A-D**).

Leaf area is an important determinant of photosynthetic efficiency, exhibiting a strong correlation between them [392]. A direct association of leaf area and photosynthetic efficiency was observed as the leaf area and photosynthetic CO_2 fixation was 1.04- and 1.03-fold higher, respectively in cv. OFSP-6 than that of WFSP-WT. Whereas in COE lines, increase in leaf area was in the range of 1.07- to 1.24-fold and photosynthetic CO_2 fixation increased up to 1.03- to 1.11-fold when compared with OFSP-6 (**Fig. 5.3 A and B)**. In TOE lines the increase in leaf area was also evident which was in the range of 1.03- to 1.20–fold higher than that of OFSP-6 and was in agreement with the previous findings *i.e.* a higher photosynthetic performance in the range of 1.13- to 1.33-fold (**Fig. 5.3 A and 5.4 A**). Photosynthetic CO_2 fixation, stomatal conductance and rate of transpiration are to some extent interlinked which was also found to be higher in both the COE (**Fig. 5.3 C and D**) and TOE lines in comparison to their wild type

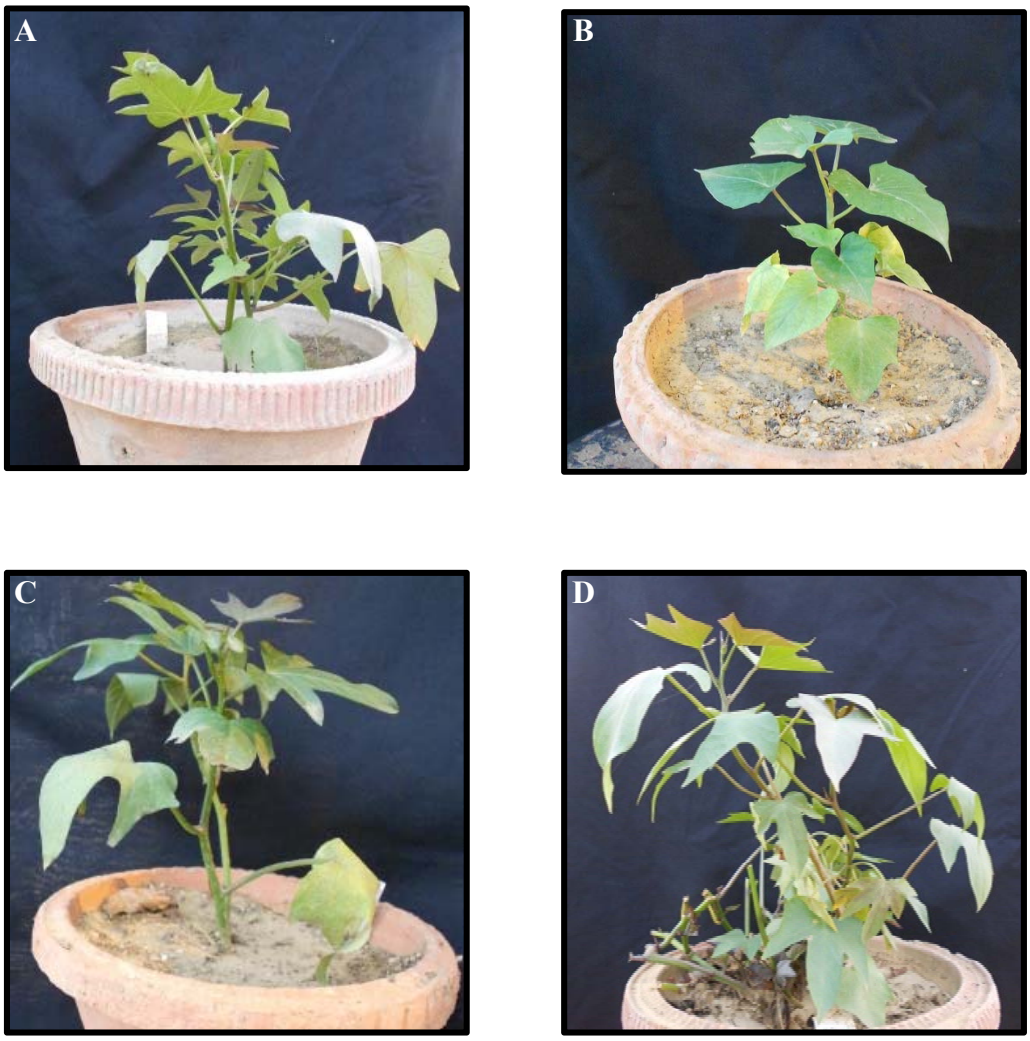

Fig. 5.1. Analysis of morphological diversity between the wild types and transgenic lines. Representative photographs displaying morphological differences in aerial parts of cv. OFSP-6 (**A**) and WFSP-WT (**B**) of wild types and COE (**C**) and TOE (**D**) transgenic lines.

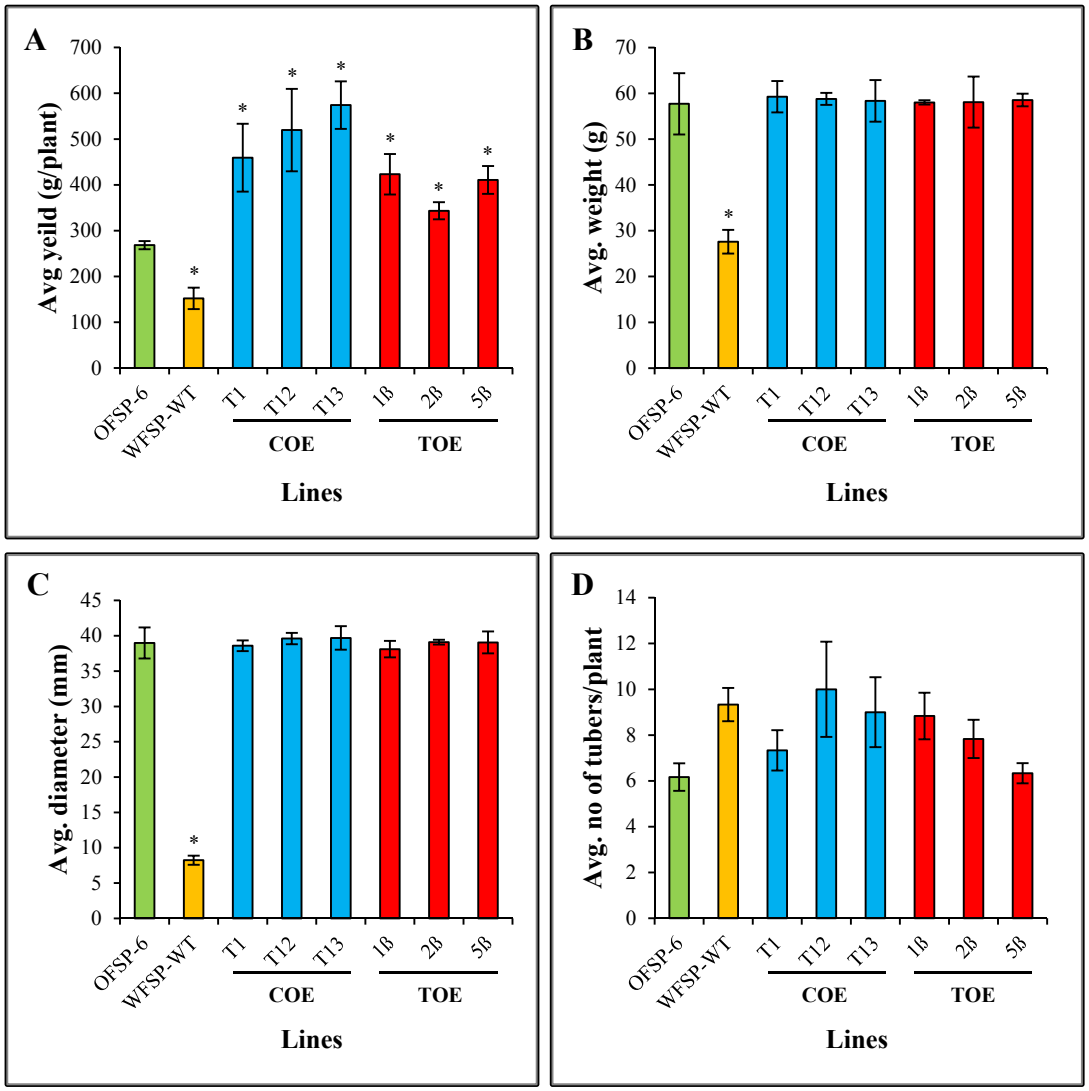

Fig. 5.2. Comparative analyses of agronomical traits. Differences in yield (**A**), weight (**B**), diameter (**C**) and number of tubers per plant was analyzed across the wild type and transgenic lines. Each bar indicates the mean values ± SE in triplicates. * indicates the level of significance at $p < 0.05$.

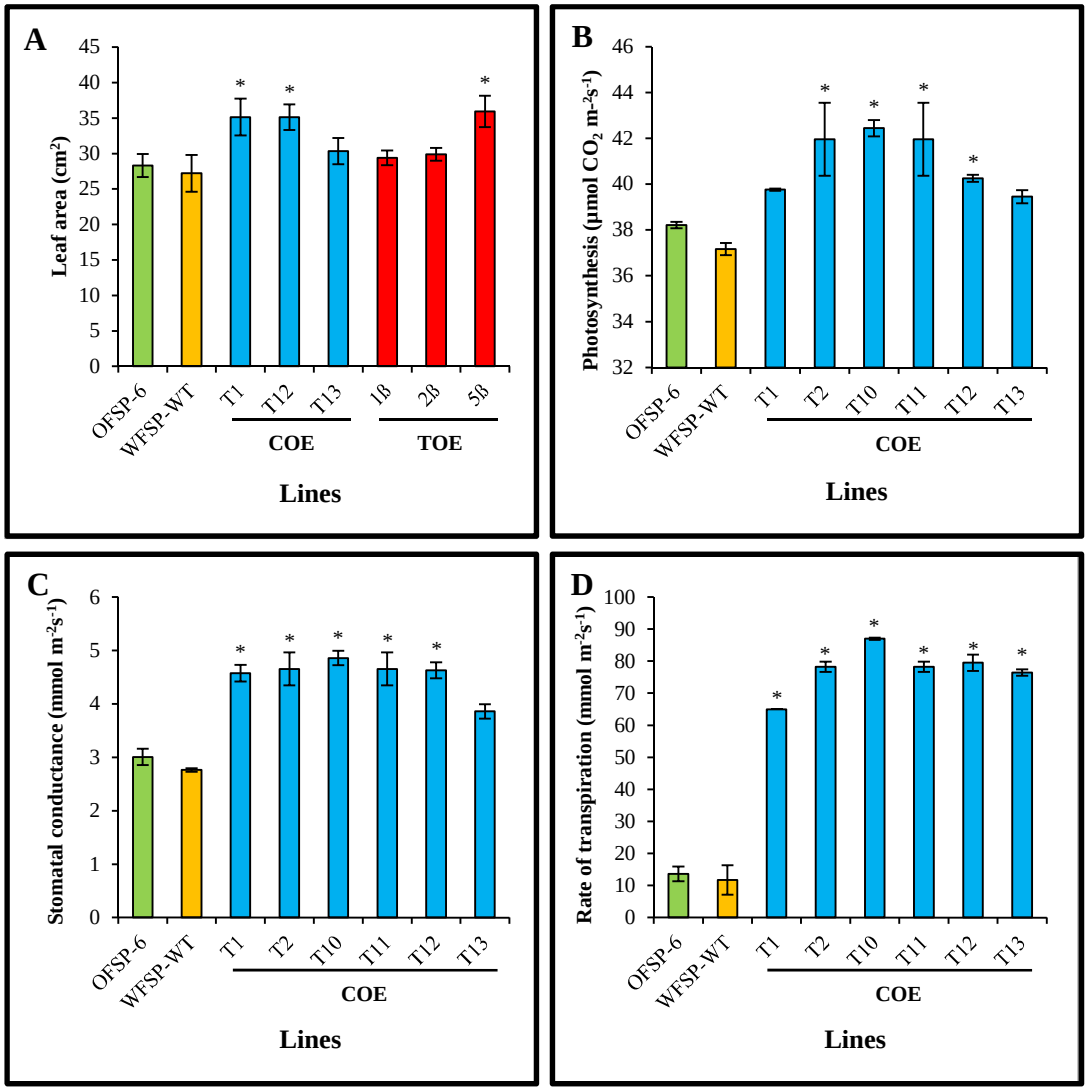

Fig. 5.3. Comparative analyses of agrophysiological traits. Leaf area of wild types (cv. OFSP-6 & WFSP-WT) and transgenic lines (COE & TOE) was analyzed (**A**), photosynthetic rates (**B**), stomatal conductance (**C**) and rate of transpiration (**D**) was compared between the two wild types and COE lines. Each bar indicates the mean values ± SE in triplicates. * indicates the level of significance at $p < 0.05$.

counterparts *i.e.* OFSP-6 (**Fig. 5.4 B and C**). To annul the possibility of any agronomic effect(s), the photosynthetic activities were also measured in four regenerated lines grown simultaneously and their photosynthetic CO_2 fixation was found to be comparable to the OFSP-6 wild type variety (**Fig. 5.4 D**).

5.3.2 Evaluation of tuber color difference

The Hunter 'L' 'a' 'b' color evaluation corroborated the visual appearance of the tuber flesh as shown in the representative photographs (**Fig. 5.5 A-D)**. Color space values demonstrated distinct colors in two contrasting sweet potato cultivars as well as in the transgenic events raised in the genetic background of OFSP-6. The 'L' (white) value for WFSP-WT (82.51) was significantly higher than that of OFSP-6 (77.31). However, in COE and TOE lines no significant change in 'L' value was observed (**Fig. 5.5 E**). A marked increase in 'a' (red) values in most of the transgenic events was observed ($p < 0.05$). The 'a' value was higher up to 4.2-fold and 1.6-fold in COE and TOE lines respectively. The 'a' (red) values of OFSP-6 (4.28) was significantly higher ($p < 0.05$) when compared with WFSP-WT (-0.6) (**Fig. 5.5 F**). Furthermore, the 'b' (yellow) values differed significantly showing a remarkable two-fold higher value in OFSP-6 (29.59) than that of WFSP-WT (14.46). The difference in 'b' value was not significant in transgenic events when compared with the corresponding wild type (cv. OFSP-6) (**Fig. 5.5 G**).

5.3.3 Biochemical and proximate analysis

5.3.3.1 Proximate composition

The nutritional status of both the sweet potato cultivars as well as the transgenic lines was also investigated. The proximate and biochemical analysis imparted a better understanding of the commonalities and diversities between the cultivars and across the transformants studied. The proximate analysis of the cultivars revealed no significant differences in terms of moisture, ash and fiber contents ($p > 0.05$). Nevertheless, both the wild type cultivars displayed high moisture content of 71.42% and 68.93% in cv. OFSP-6 and WFSP-WT, respectively. The difference in moisture content among the transgenic lines was not found to be significant even as compared to OFSP-6. However, all the transgenic lines maintained higher moisture content in comparison to their wild type counterpart (**Table 5.1**). The ash and fiber contents were higher in cv. OFSP-6 (4.7% and 2.35%, respectively) as compared to WFSP-WT (4.46% and 2.31%, respectively)

which was not different considerably throughout the transformants. The ash and fiber contents in the transformants were respectively in the range of 4.67- 4.83% and 2.33-2.36% (**Table 5.1**).

5.3.3.2 Assessment of increase in total protein content in transgenic events

The fundamental purpose of genetic transformation in sweet potato was to invariably enhance its protein content**.** Total protein content was evaluated in all the transgenic events as well as in the wild types. Micro-kjeldahl analysis revealed approximately 10-83% increase in the protein contents in COE lines when compared with its wild type counterparts (OFSP-6) (**Fig. 5.6 A; Table 5.2**). Whereas, in TOE lines the increase in protein content was observed to be in the range of ~18-70% which further corroborated the higher transgene expression in the COE lines than that of TOE lines (**Fig. 5.6 B; Table 5.3**). Interestingly, total protein content (4.85 g/100g) of OFSP-6 was considerably higher than that of WFSP-WT (2.93 g/100g). These results also imply that OFSP-6 contains more nitrogenous substances as compared to WFSP-WT (**Fig. 5.6 A; Table 5.2**). To nullify the possibility of any effect of micropropagation or *in vitro* growth condition on the increase in the protein content, micro-kjeldahl analysis was also carried out in four regenerated lines which revealed almost equal protein contents in those lines when compared to OFSP-6 (cv. SP-6) (**Fig. 5.6 C; Table 5.4**).

5.3.3.3 Assessment of amino acid content

Amino acids are the major contributors of nitrogen in proteins. The overall content of nitrogen in dietary protein is about 16% by weight; therefore, nitrogen metabolism is often considered to be tantamount with protein metabolism. The expected increase in protein contents of the transgenic lines impelled to study the transgene effect at amino acid level. The total and individual amino acids were analyzed by ninhydrin based calorimetric method and GC-MS, respectively. Nonetheless, elevation in the level of total free amino acids was observed in all the transgenic lines but in few of the higher AmA1 expressing lines, it was found to be significantly enhanced ($p < 0.05$) (**Fig. 5.7**). The amino acid level in both the wild types OFSP-6 and WFSP-WT was also in concordance with the findings of the micro-kjeldahl analysis (**Fig. 5.6**).

This analysis was further extended and analyzed via absolute quantification of metabolites including amino acids using GC-MS to understand the impact. In this analysis, amino acids were evaluated taking the most promising COE and one TOE line. Only those amino

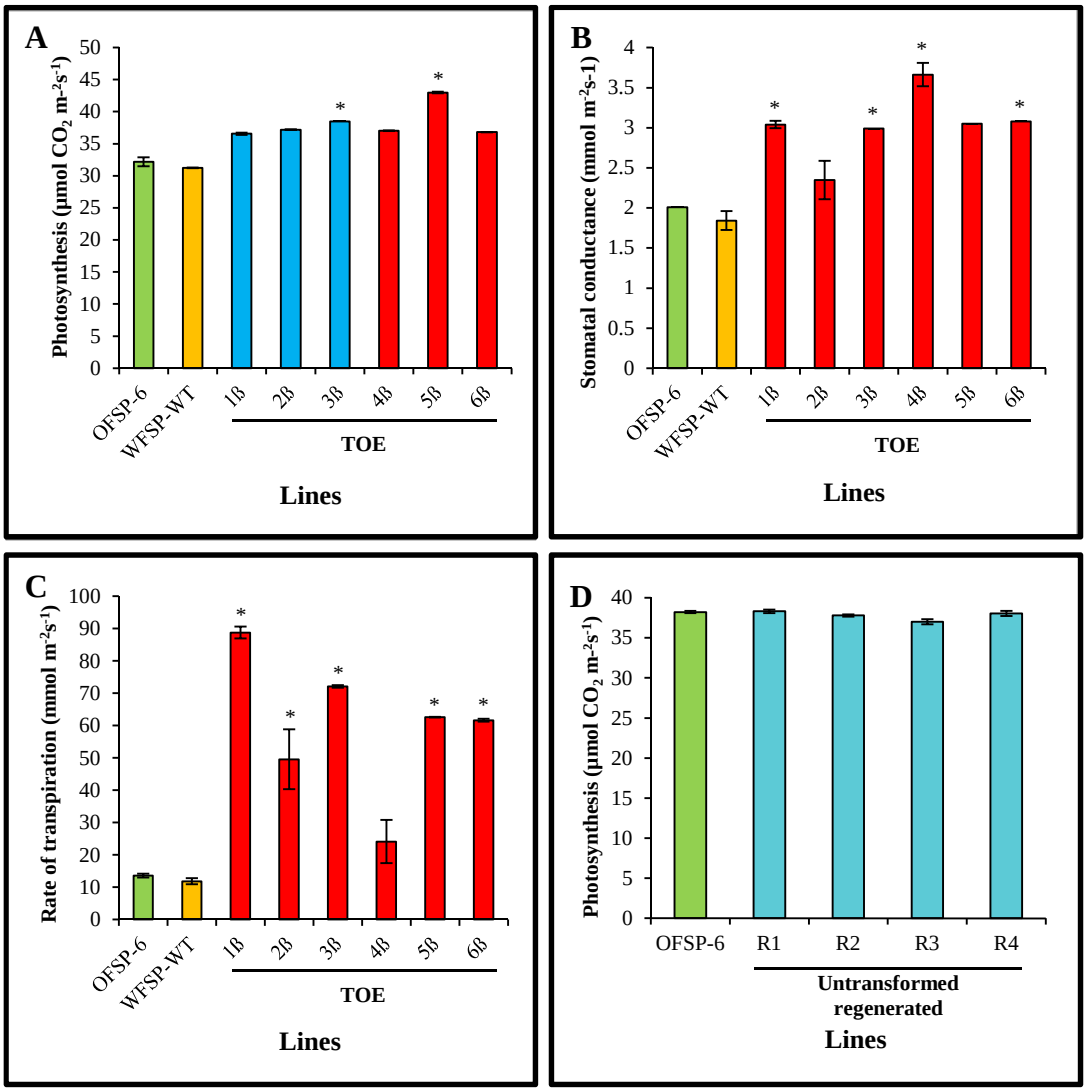

Fig. 5.4. Comparative analyses of photosynthetic performance. Photosynthetic rates (**A**), stomatal conductance (**B**) and rate of transpiration (**C**) was compared between the two wild types (OFSP-6 & WFSP-WT) and TOE lines. To annul the possibility of any agronomic effect(s), the photosynthetic activities were also measured in four regenerated lines (**D**). Each bar indicates the mean values ± SE in triplicates. * indicates the level of significance at $p < 0.05$.

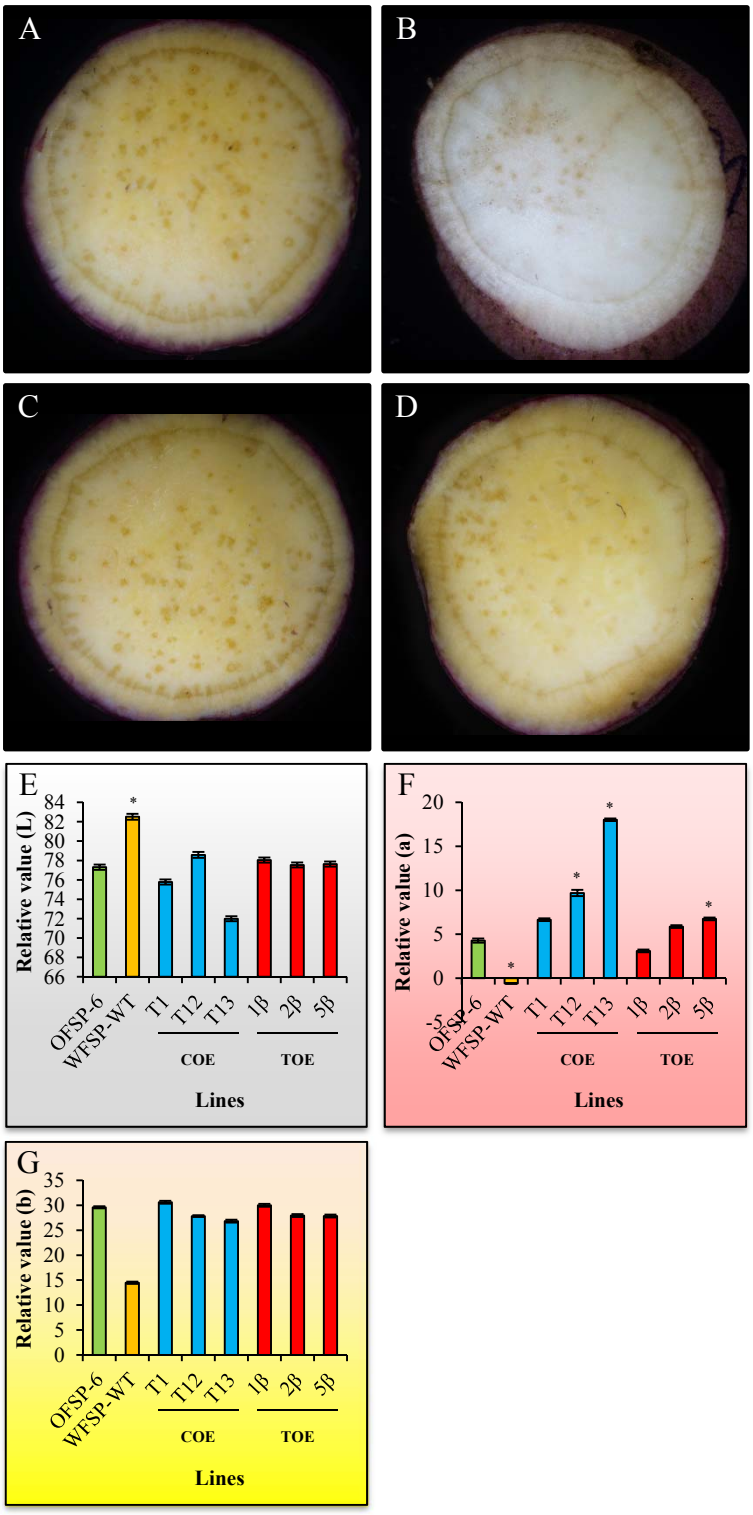

Fig. 5.5. Evaluation of color differences. Cross sections of tubers displaying color differences in OFSP-6 (**A**), WFSP-WT (**B**), COE (**C**) and TOE (**D**) lines. The relative Hunter 'L' 'a' 'b' values were determined (**E-G**). Data represent mean values ± SE of three measurements. * indicates the level of significance at $p < 0.0\,5$.

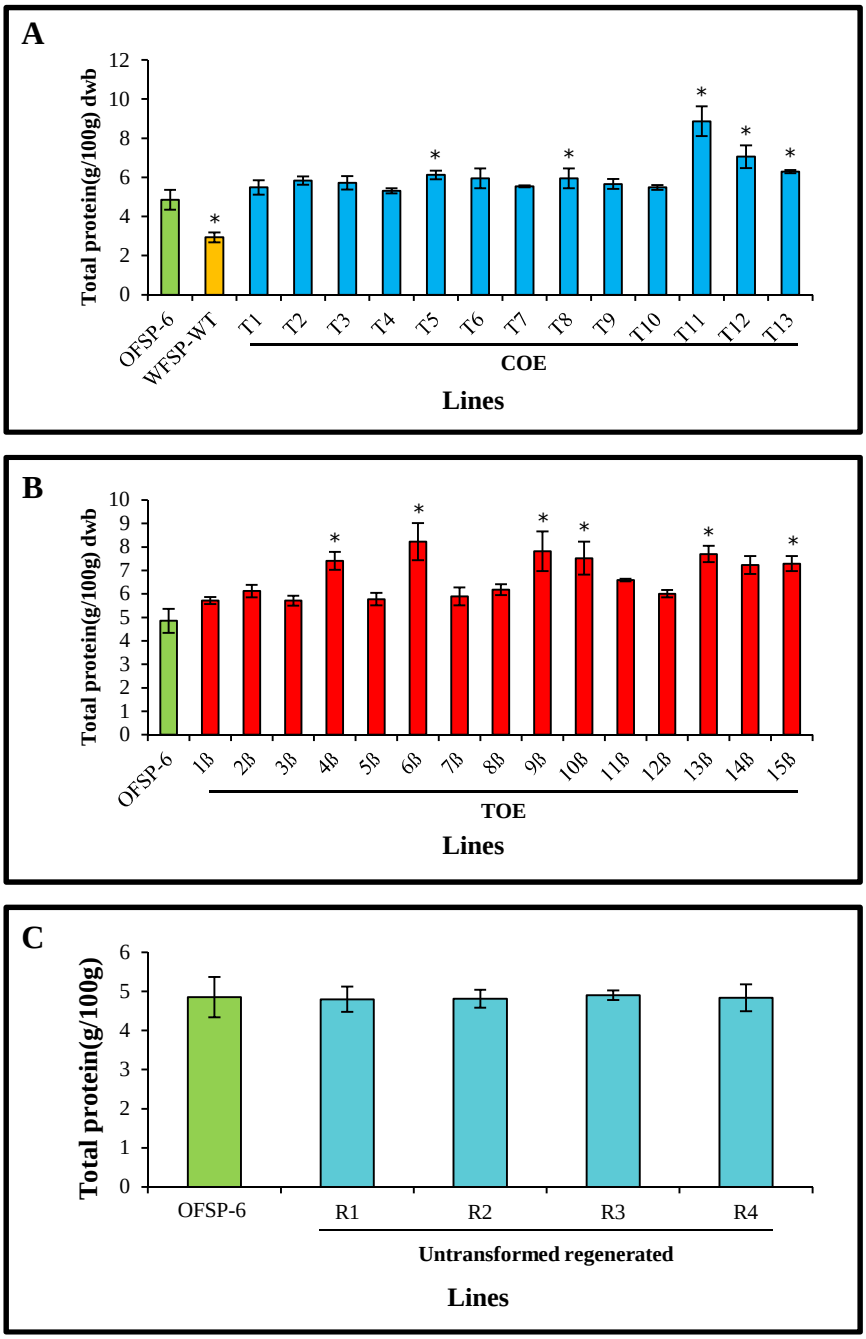

Fig. 5.6. Evaluation of total protein content by micro-kjeldahl analysis. Total protein content was compared between the wild types Vs COE (**A**) and TOE (**B**) lines. Protein content was evaluated from five different harvests. To nullify the possibility of any effect of micropropagation or *in vitro* growth condition total protein content was also determined in regenerated lines (**C**). Data represent mean values ± SE of five measurements. * indicates the level of significance at $p < 0.05$.

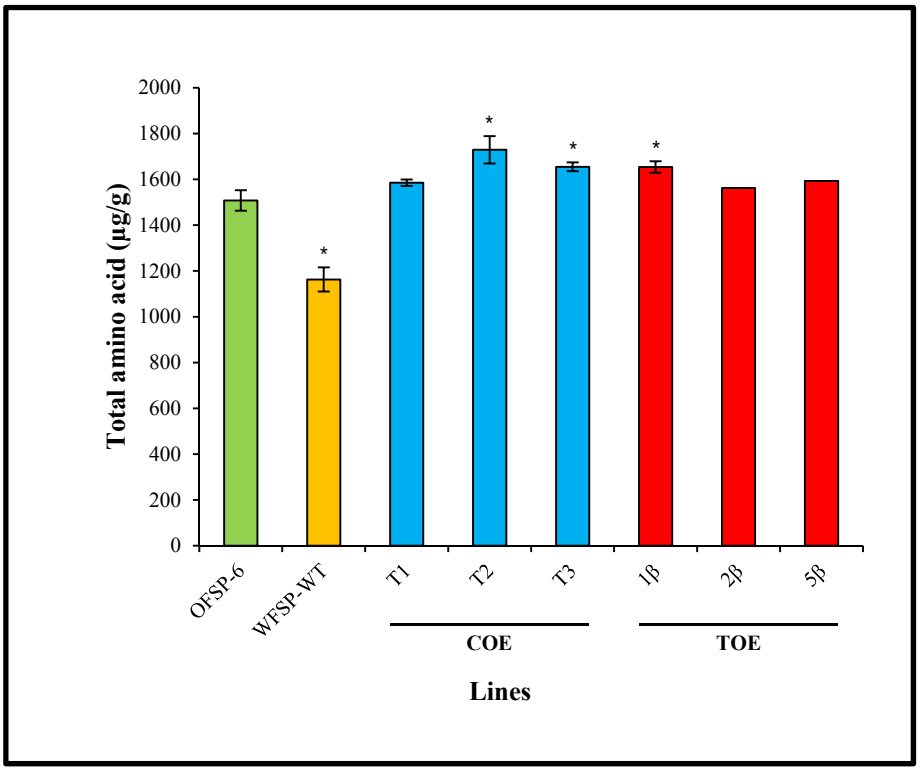

Fig. 5.7. Assessment of amino acid content. Total content of amino acids was evaluated in wild types (cv. OFSP-6 & WFSP-WT) and transgenic lines by ninhydrin based calorimetric method. Data represent mean values ± SE of three measurements. * indicates the level of significance at $p < 0.05$.

Table 5.1. Proximate composition of sweet potato lines.

Cultivars/Lines	Moisture (%)	Ash (%)	Fiber (%)
OFSP-6	71.42 ± 0.33	4.7 ± 0.05	2.35 ± 0.15
WFSP-WT	68.93 ± 0.26	4.46 ± 0.26	2.31 ± 0.09
T1	72.34 ± 0.42	4.67 ± 0.37	2.34 ± 0.04
T12	73.88 ± 0.64	4.73 ± 0.05	2.36 ± 0.10
T13	73.77 ± 0.22	4.76 ± 0.11	2.35 ± 0.01
1ß	72.28 ± 0.36	4.83 ± 0.28	2.33 ± 0.03
2ß	72.33 ± 0.26	4.77 ± 0.40	2.34 ± 0.03
5ß	72.75 ± 0.09	4.73 ± 0.50	2.35 ± 0.04

Values are presented as means ± S.E. (n=3) of a composite sample of four to eight tubers.

Table 5.2. Micro-kjeldahl analysis of constitutive (COE) lines

Lines	Total protein(g/100g)	% increase in trangenic lines
OFSP-6	4.85 ± 0.51	
WFSP-WT	2.93 ± 0.25	
T1	5.49 ± 0.37	13.01
T2	5.84 ± 0.21	20.22
T3	5.72 ± 0.34	17.78
T4	5.31 ± 0.12	9.37
T5	6.13 ± 0.20	26.19
T6	5.95 ± 0.50	22.55
T7	5.54 ± 0.05	14.17
T8	5.95 ± 0.50	22.62
T9	5.66 ± 0.25	16.61
T10	5.48 ± 0.12	12.97
T11	8.87 ± 0.76	82.68
T12	7.06 ± 0.57	45.42
T13	6.3 ± 0.08	29.76

Table 5.3. Micro-kjeldahl analysis of tuber specific (TOE) lines

Lines	Total protein(g/100g)	% increase in trangenic lines
OFSP-6	4.85 ± 0.51	
1ß	5.72 ± 0.15	17.78
2ß	6.13 ± 0.26	26.19
3ß	5.72 ± 0.21	17.78
4ß	7.41 ± 0.38	52.63
5ß	5.78 ± 0.26	18.98
6ß	8.23 ± 0.78	69.46
7ß	5.89 ± 0.38	21.38
8ß	6.18 ± 0.23	27.39
9ß	7.82 ± 0.84	61.04
10ß	7.53 ± 0.70	55.03
11ß	6.59 ± 0.05	35.81
12ß	6.01 ± 0.15	23.79
13ß	7.7 ± 0.35	58.64
14ß	7.23 ± 0.38	49.03
15ß	7.29 ± 0.32	50.23

Table 5.4. Micro-kjeldahl analysis of regenerated lines

Lines	**Total protein(g/100g)**
OFSP-6	4.85 ± 0.51
R1	4.79 ± 0.32
R2	4.81 ± 0.23
R3	4.9 ± 0.12
R4	4.84 ± 0.34

acid levels were considered for this analysis which was co-eluted at or around same retention time (RT). Analysis of the pool sizes of several amino acids revealed a significant increase in amino acids, notably lysine, leucine, methionine, phenylalanine and valine, which are otherwise limited in sweet potato. However, threonine content was unpredictably found to be very low in both the transgenic lines. Increase in the level of non essential amino acids such as alanine, aspartic acid, glutamine, asparagine and serine further indicated that the augmentation of the essential amino acids is not at the expense of other amino acids (**Fig. 5.8 A-L**). These changes were notably more prominent in transgenic events, exhibiting a higher level expression of AmA1. These findings further corroborated the calorimetry based analysis of amino acids as well as the comprehensive increase in total proteins by micro-kjeldahl analysis.

5.3.3.4 Analysis of carbohydrates

To understand the nutritional diversity between the two wild type varieties of sweet potato as well as the transgene mediated changes in the fundamental metabolism, an extensive biochemical analyses was carried out. Biochemical analyses further revealed that the total carbohydrate content of WFSP-WT was significantly higher (14.28 mg/g) as compared to OFSP-6 (12.29 mg/g). However, no considerable change was observed across the transgenic lines. (**Fig. 5.9 A**).

The reducing sugar was also higher in WFSP-WT (4.47%) than that of OFSP-6 (2.76%) and intriguingly, it reduced up to half in transgenic lines when compared with OFSP-6 as the reducing sugar across the transgenic lines was 1.28-1.67% (**Fig. 5.9 B**). However, the starch content was higher in case of OFSP-6 (55.83 g/100g) as compared to the WFSP-WT (43.35 g/100g). Both the COE and TOE lines followed the same trend like OFSP-6 as the starch content was elevated in all the transgenic lines as compared to their wild type counterparts (OFSP-6) (**Fig. 5.9 C**). Interestingly, out of all the transgenic events investigated, the highest starch content in T12 (69.67 g/100g) having the lowest reducing sugar content (1.44%). Albeit, the reverse was off course not witnessed in case of lower starch categories. Therefore, the enhanced carbohydrate and reducing sugar might be the product of starch and cellulose degradation.

5.3.3.5 Quantitative determination of water holding capacity

WHC signifies the amount of water retained by a known weight of the sample and to some extent related with the protein and carbohydrate contents specially the starch content of the food sample [393, 394]. A direct correlation was observed between the starch and protein contents as WHC was found to be higher in cv. OFSP-6 than that of WFSP-WT. Intriguingly, total starch, total protein and WHC were 1.20-, 1.29- and 1.07-fold higher, respectively in OFSP-6 (**Fig. 5.9 D**). Moreover, in COE and TOE lines, WHC was quite higher when compared with OFSP-6. WHC was observed to be the highest in T13 line and in comparison to OFSP-6 total starch, total protein and WHC were 1.20-, 1.29- and 1.44-fold higher, respectively.

5.3.3.6 Evaluation of storage stress response

To elucidate the impact of storage stress on carbohydrates, especially starch and cellulose, the rate of degradation was investigated. The disparity in the status of total carbohydrate, reducing sugar and starch contents impelled to examine the degradation pattern of starch in wild types as well as in the transgenic lines, during post-harvest storage. The starch content showed progressive declined till 105-d, and about 41% loss was observed in WFSP-WT. However, 29% decline in the starch content was observed until 105-d in OFSP-6 (**Fig. 5.10 A**). In transgenic lines the effect of storage stress was evaluated taking one best line from each category. At the initial stage (0-d) the starch content was almost equal in both the lines and it was imperative to examine the effect across the transgenic lines as well. The linear decline was not observed in transgenic lines and moreover, the starch degradation was comparatively slower than the wild types. In COE line, the decline in starch content was 23% whereas in TOE line it was 26% until 105-d (**Fig. 5.10 A**).

The alteration in cellulose content showed a similar trend. However, the cellulose content in both the cultivars decreased progressively until 105-d during the storage but degradation was more prominent in WFSP-WT. The degradation of cellulose was 44% in WFSP-WT as against 35% in OFSP-6. In COE and TOE lines, the decline in cellulose content was found to be 33% and 34%, respectively (**Fig. 5.10 B**).

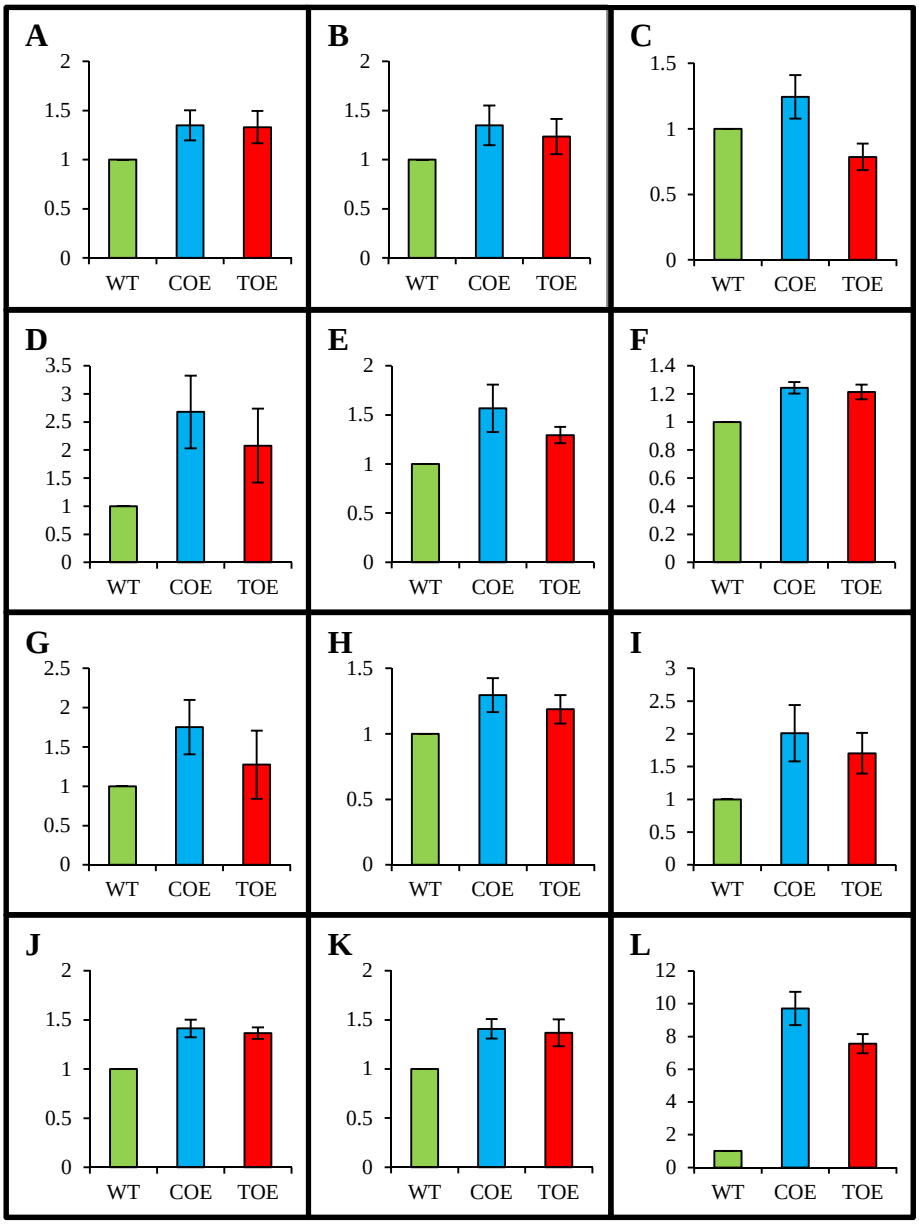

Fig. 5.8. Comparison of elevation in the level of total free amino acids by GC-MS. Comparison of amino acid levels in mature AmA1 tubers with those in tubers of wild-type (**A**) L-Leucine (**B**) Serine (**C**) L-Aspartic acid (**D**) Valylvaline (**E**) L-Aspartic acid (**F**) Phenylalanine (**G**) L-Asparagine (**H**) Alanine (**I**) Glutamine (**J**) L-Lysine (**K**) L-Valine and (**L**) L-Methionine. Data are normalized to the mean response calculated of each replicate taking ribitol as internal standard. Y axis represents the value of relative response ratio.

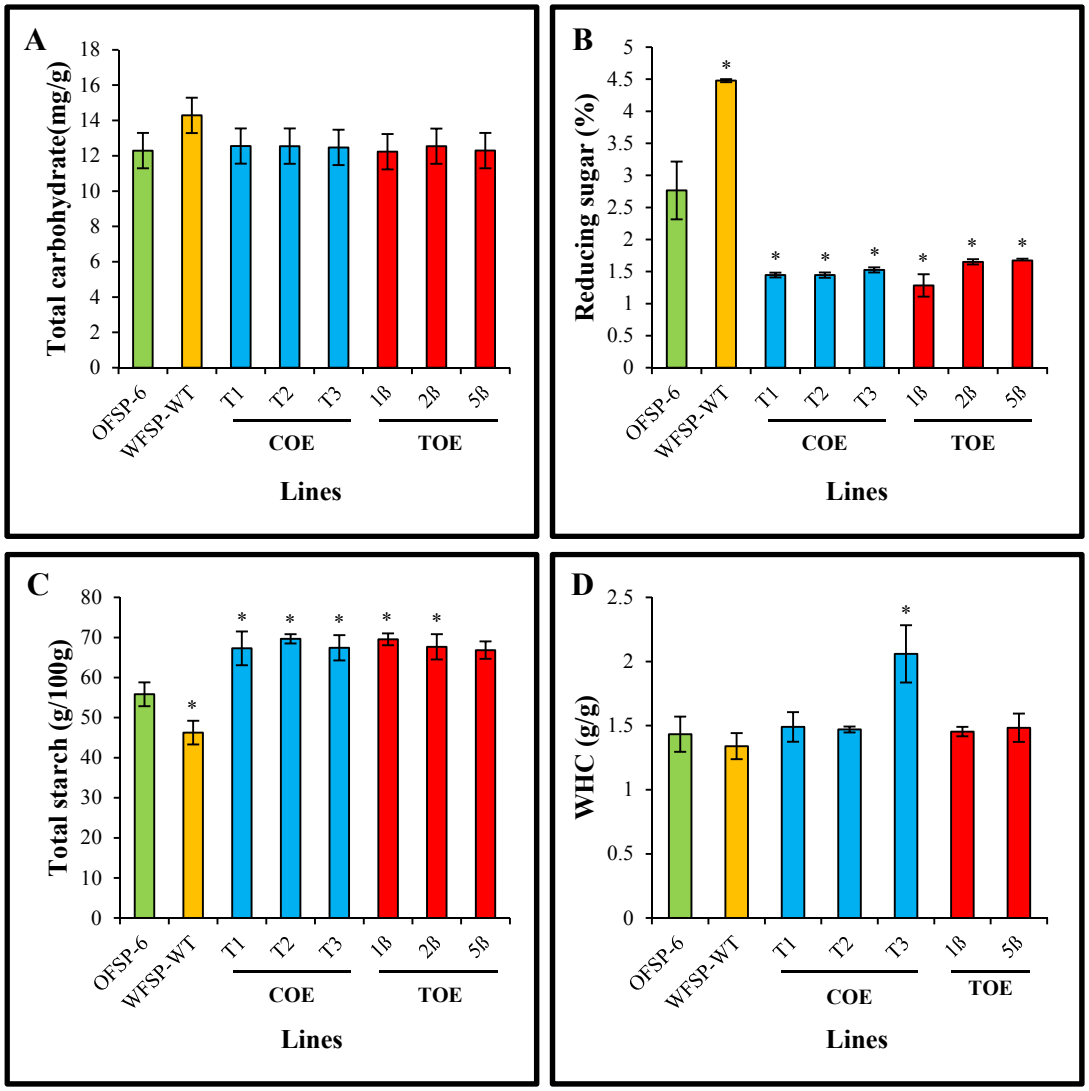

Fig. 5.9. Comparative biochemical analyses. Comparative biochemical analyses ⁎ of wild types (OFSP-6 & WFSP) were performed. Total carbohydrate (**A**), reducing sugar (**B**), and starch content (**C**) were determined on dry basis. The quantitative determination of Water holding capacity (WHC) is shown (**D**). Data represent mean values ± SE of three independent measurements. * indicates the level of significance at $p < 0.05$.

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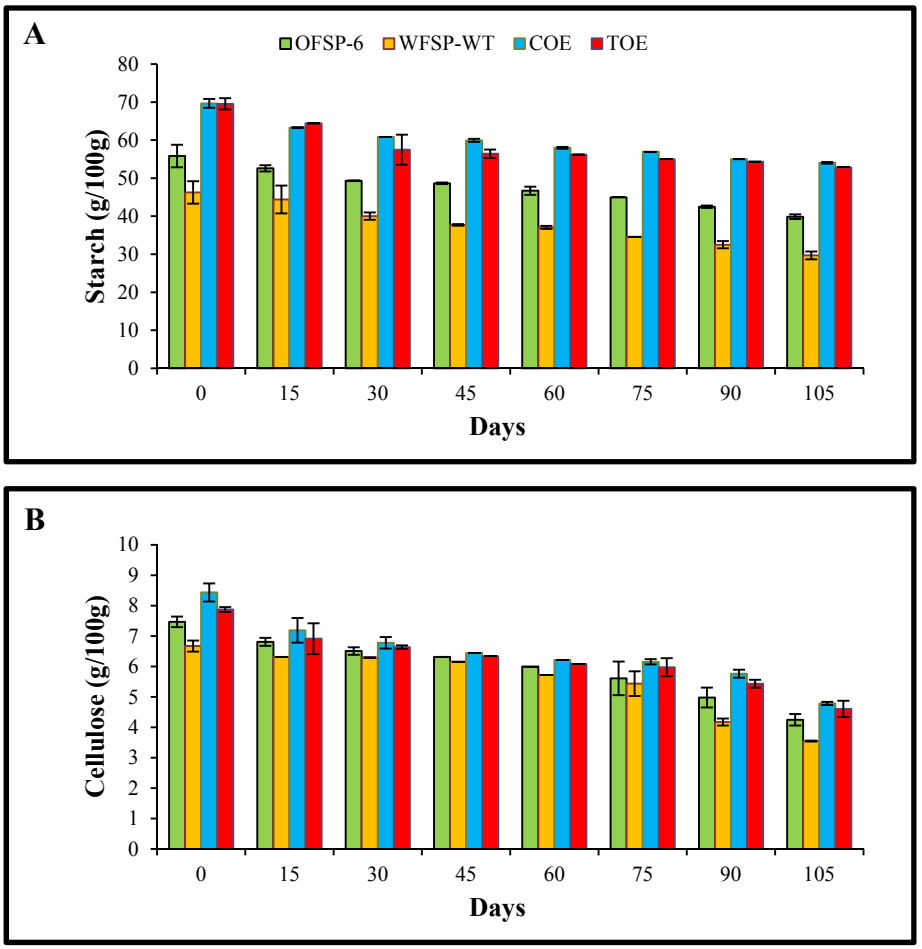

Fig. 5.10. Evaluation of storage stress response. Tubers were stored at room temperature and sampled at every 15-d interval until 105-d. The starch (**A**) and cellulose (**B**) contents were measured on dry basis at a succession of storage time points in wild type (OFSP-6 & WFSP-WT) and transgenic (COE & TOE) lines. Data represent mean values ± SE of three measurements

5.3.3.7 Structural and chemical characterization

To investigate as well as to negate the possibility of any modification in starch, SEM analysis was carried out from the composite tuber samples of transgenic as well as the wild types. Apparently the crystal structure of starch showed no indication of modification in the transgenic events as compared to the wild types. However, the difference in shape of starch crystals due to the difference of genetic constitution and /or varietal difference was clear between the wild types OFSP-6 and WFSP-WT (**Fig. 5.11 A-D**). These findings were further in agreement with FT-IR spectra obtained from both the wild types and the transgenic lines. The FT-IR spectroscopy was used to confirm the presence of functional groups in the chemical structure of sweet potato composite samples used to verify any modification(s). FT-IR spectra in the four major regions help in successive elucidation and categorization of the key bands. More than a few characteristic absorption bands were observed in **Fig. 5.12 A**, such as C–O, C–H, and hydroxyl groups stretching vibration. Compared with the native samples, the spectrum of sweet potato samples showed no new spectra in the transgenic lines further negating any modification. Even the wild type variety showed no difference in the characteristic bands obtained (**Fig. 5.12 A**). X-ray diffraction (XRD) pattern of the samples also did not reflect any modification in transgenic tuber sample (**Fig. 5.12 B**). However, the overall diffraction pattern was not discrete since it represented not only the diffraction of starch but a global pattern of X-ray diffraction from the composite samples.

5.4 Discussion

The purpose of this study was to examine the effect of the transgene on the agrophysiological traits as well as on the overall metabolism. At this juncture, two key questions arose were, whether there was any metabolic shift due to the transgene introduction and if yes, in which way it was. To address this question, a multivariate analysis of transgenic events was carried out simultaneously with the wild type. In addition, to abrogate the possibility of natural and/or somaclonal variations amongst different transgenic lines a close relative (WFSP-WT) of OFSP-6 (cv. SP-6), was also included in this study. All the transgenic lines were grown in parallel to both the wild types in identical conditions and their agrophysiological performance was monitored. Most of the transgenic events showed a greater yield than the wild types (**Fig. 5.1**). Increased tuber yield is closely associated with the photosynthetic carbon metabolism,

which is considered to be a crucial factor for plant growth and productivity [395]. We, therefore, investigated the photosynthetic efficiency of transformants *vis-à-vis* the wild types which showed superior efficacy of COE and TOE transgenic lines. Furthermore, a strong correlation was found between the increased leaf areas, photosynthetic rate (**Fig. 5.2-5.4**) and total protein content (**Fig. 5.6**). Several earlier reports revealed that the increase in protein biosynthesis is a consequence of increased rate of photosynthesis, which is eventually to be a crucial factor for higher yield [396, 397]. It is increasingly clear that the tuberization is a complex process involving various metabolic cues that include massive accumulation of starch and proteins. Tuber as a storage organ acts as a sink and competes for the available photo-assimilates. The allocation of photoassimilates is an important criterion for plant productivity based on the harvest index. In tuberous crop, a higher harvest index indicates an efficient diversion of photoassimilates sink [61, 62]. Transgene introduction in sweet potato resulted into the increase in total proteins revealed by micro-kjeldahl analysis (**Fig. 5.6**). Higher content of total free amino acids as well as individual amino acids in transgenic events further corroborated the increase in total protein (**Fig. 5.7 and 5.8**). In addition, the increase in amino acids was not restricted to essential amino acids which further contradicts several reports regarding the introduction of seed storage proteins wherein introduction of these genes in target plants has often resulted in an increase in one of the amino acids at the expense of others, leading to an imbalance of the amino acid profile in transgenic crops [398-400]. Nonetheless, the expression level of AmA1 in transgenic tubers was not high enough to be directly correlated with the protein increase, though, comparative proteome analysis showed a positive correlation with the finding of biochemical analysis and *vice-versa* (**Fig. 4.15 B; Section 4.3.6.2**). The *de novo* synthesis and accumulation of unique proteins and/or quantitative changes in the expression level or rebalancing of proteins as a result of AmA1 expression could be the reason behind the increase in total protein content as revealed by micro-kjeldahl and 2-DE analyses. Earlier it has been established that the synthesis of seed storage protein depletes free amino acid in the storage organ that usually leads to an increase in the rate of photosynthesis [396]. It is likely that AmA1, a seed storage protein, when introduced in sweet potato tuber might lead to the depletion of the endogenous free amino acid pool for its synthesis and accumulation. It is expected that the depletion of endogenous free amino acids in the transgenic tubers is then sensed by the photosynthetic machinery, causing an

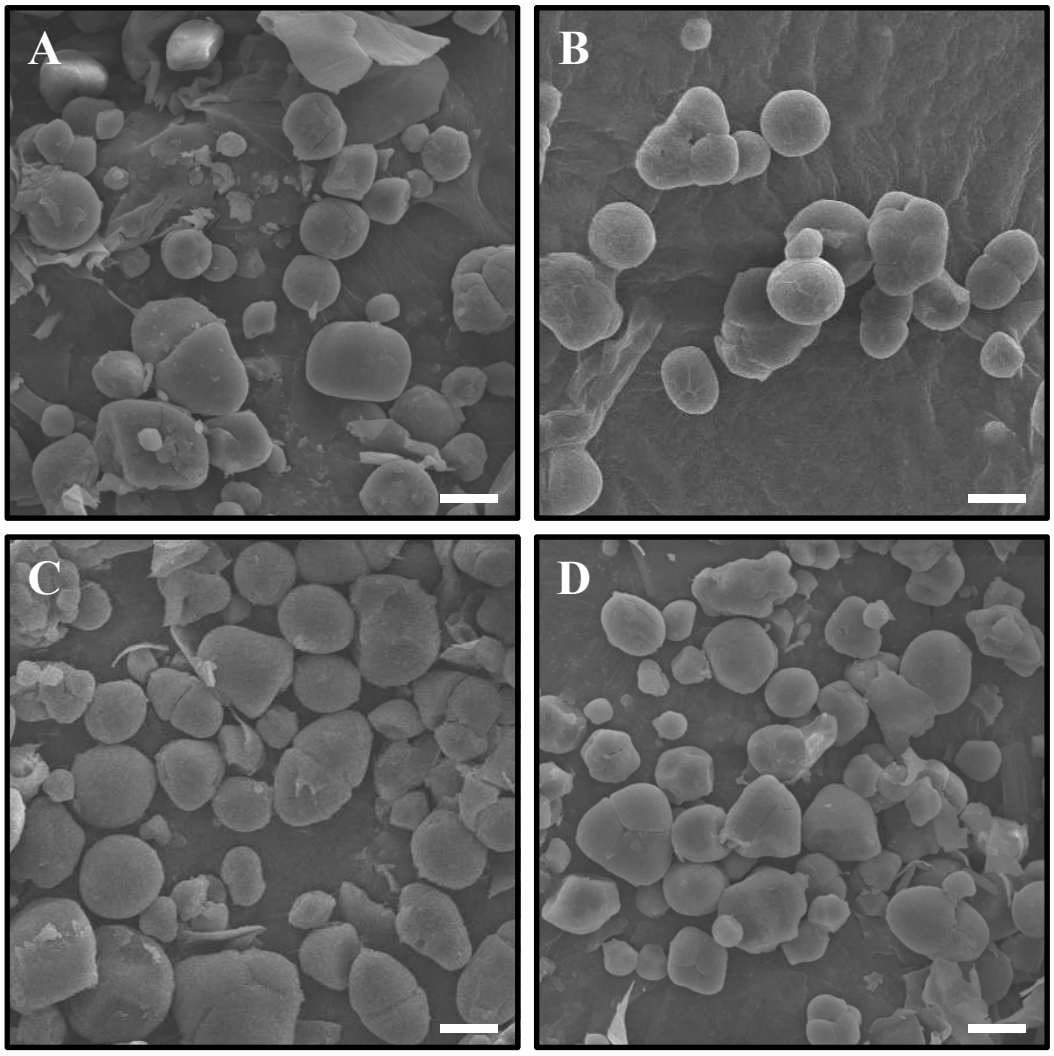

Fig. 5.11. Structural characterization of starch. Structure of starch crystals was visualized by SEM in OFSP-6 (**A**), WFSP-WT (**B**), and COE (**C**) and in TOE (**D**) lines. Bar represents 10 μm of magnification

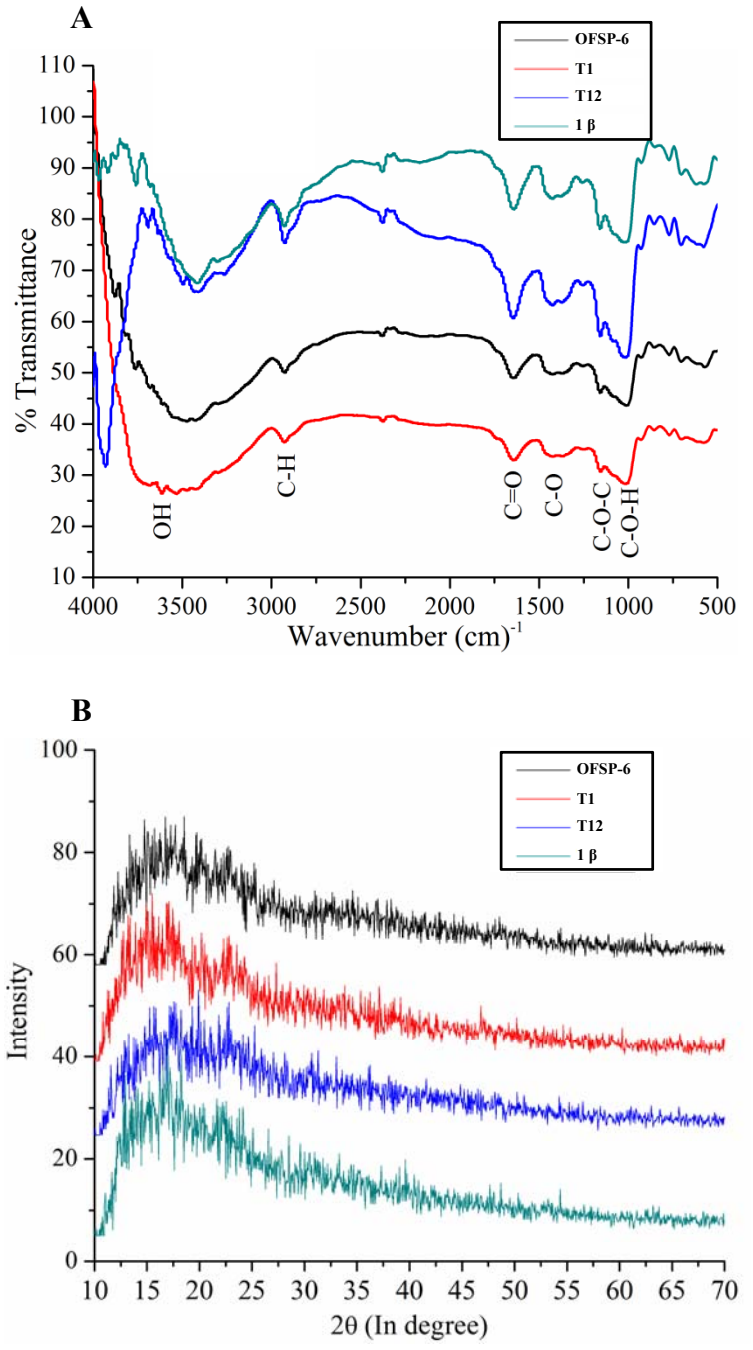

Fig. 5.12. Chemical characterization of composite samples of sweet potato. Composite samples of sweet potato wild type (cv. OFSP-6), COE (T1 & T12) and TOE (1β) lines were examined for any possible chemical modifications by FT-IR (**A**) and XRD (**B**).

increase in the rate of photosynthesis. Increase in the protein content via transgene approach had also showed a profound effect on the rate of photosynthesis previously [113, 121].

The proximate and biochemical analysis imparted commonalities along with diversities between the wild type and transgenic lines studied. The proximate analysis of the wild types and transformants revealed no significant difference in case of moisture, ash and fiber contents ($p > 0.05$). Nevertheless, the wild type cultivars displayed high moisture content of 71.42% and 68.93% in OFSP-6 and WFSP-WT, respectively. Even the highest moisture content displayed by the transgenic line was 73.88%. The moisture content was found to be well within the range of 60-84%, as reported for many sweet potato cultivars [401]. The high-moisture content is an indicator of low dry matter and lower storage quality in tubers. The differences in the moisture content among different cultivars may possibly be attributed to either the difference in the genetic composition and/or the agro-climatic conditions [34]. The ash and fiber contents were higher in OFSP-6 (4.7% and 2.35%, respectively) as compared to WFSP-WT (4.46% and 2.31%, respectively) which was well within the range reported earlier in different cultivars [402]. In addition, the transgenic lines showed no significant differences from its wild type counterpart OFSP-6 (**Table 5.1**). In most sweet potato cultivars, the percentage of dry matter is reported up to 44%, out of which approximately 90% is carbohydrate [11]. The present study revealed a considerably higher carbohydrate content in WFSP-WT (14.28 mg/g) as compared to OFSP-SP (12.29 mg/g). However, no considerable change was observed across the transgenic lines since the total carbohydrate content was found to be equivalent to OFSP-6. The reducing sugar content was also higher in WFSP-WT (4.47%) than that of OFSP-6 (2.76%); however, starch content was higher in case of OFSP-6 (55.83 g/100g) as compared to the WFSP-WT (43.25 g/100g). Transgenic lines virtually showed a similar trend as the starch content was elevated in all the transgenic lines as compared to their wild type counterpart (cv. OFSP-6) (**Fig. 5.9**). The disparity between the starch contents and the status of carbohydrate and reducing sugar suggests a possible degradation of starch and cellulose, wherein starch hydrolyzes and get converted into reducing sugars during storage [34]. This further necessitated the analysis of the degradation pattern of starch in wild types and transgenic lines. Post-harvest decline in starch content among different cultivars might be due to differential metabolism, especially the respiratory intensity and the increase in amylase activity during the storage [89]. The transgenic lines maintained a constant high starch and cellulose content during storage stress and even the extent of degradation was

found to be lower than that of wild types. The degradation of starch and cellulose altogether indicates better adaptability of the transformants during storage stress. The overexpression of AmA1 may possibly have provided a better adaptability to sustain the basal metabolism in transgenic tubers in such a way that the degradation of stored carbohydrate source (starch and/or cellulose) is lesser in demand. Moreover, it seems likely that the increase in nitrogen storage in sink tissue may result in alteration of total biomass production. Nitrogen storage and carbohydrate metabolism are intimately related. Therefore, it can be postulated that the carbohydrate distribution within the plant might be affected by the nitrogen supply, which strongly influences the processes of carbon metabolism, and thus nitrogen status has a great impact on the postharvest performance of plants [403].

Sweet potato is a starch rich crop, therefore it is imperative to investigate any transgene mediated unintended effects in starch. Any modification in starch can alter its crystal structure as improving maize protein quality that maintained high lysine content had invariably changed the structure of native starch [404]. Therefore, the structure of starch was visualized by SEM analysis which suggested no sign of alteration. Several reports with reference to metabolome studies are based on NMR and FT-IR. FT-IR had also been employed as probes to investigate the process-induced changes and to measure food quality. These studies include an immense range of investigations to safety-intended studies [405, 406]. The principle of FT-IR lies in the fact that, when a sample is introduced with electromagnetic radiations, chemical bonds at specific wavelengths absorb these radiations and vibrate. These absorptions and subsequent vibrations can then be correlated to single bonds or functional groups of a molecule for the identification of unidentified compounds. The major region of importance is in the mid-IR, which is usually defined as 4000–600 cm^{-1} and on the basis of chemical information content and spectral richness (*i.e.* numbers of clearly defined peaks) any chemical signature including the newly emerged can be interpreted. In relation to biological applications, the mid-IR can be further broken down into what are termed spectral windows of interest, where strong absorption bands are able to be directly related to specific compounds [407]. Accomplishment of a credible finding by these methods relies on efficient utilization of data from the spectra obtained and its concomitant analysis through a plausible classification method. In this study, FT-IR analysis supported similar chemical signatures in transgenic events than that of wild type thereby negating the

possibilities of any modifications. The XRD pattern also advocated in support of SEM and FT-IR analysis.

In summary, introduction of AmA1 has a profound effect on the overall nutritional status of sweet potato. The introduction of transgene has not only improved the total protein content, but also its agrophysiological and biochemical characteristics without a major metabolic shift. Nonetheless, the total carbohydrate content was not affected but the slower degradation of starch and cellulose increased the post-harvest durability of the transgenic tubers thereby increasing its consumer acceptability.