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Improvement of *in vitro* regeneration frequency, polyphenolic and antioxidant profile of Strawberry (*Fragaria ananassa* Cv. Chandler) via indirect organogenesis

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Abstract

The Strawberry (*Fragaria ananassa*) is a fleshy fruit crop from the Rosacea family, considered one of the most economically significant plants. Strawberry is an accessory fruit crop that has become known for its attractive red color, sweet scent and taste, and high fiber, nutrient, and sugar content. Strawberry contains a significant portion of phenolic, flavonoids, Vitamin C, and potent antioxidants. To use *in vitro* approaches in berry crops, efficient regeneration protocols must be established. The main intent of the current study was to establish an effectual regenerative protocol and evaluate the regeneration efficacy of Thidiazuron (TDZ) and the antioxidant profile of *Ex-vitro* and *in vitro* strawberry plants. *In vitro*-derived stem explants were inoculated on Murashige and Skoog (MS) media augmented with diverse Plant growth regulators (PGRs) for initiation of callus. The induced calli were further subcultured on MS media containing different concentrations of TDZ for shoot stimulation. *In vitro*-derived shoots were further shifted to the rooting medium. The Polyphenolic content was determined for regenerated *ex-vitro* and *in vitro* plants. MS media comprising NAA (0.5mg/L) + 2, 4-D (0.5mg/L) induced the highest callus formation. MS media added with TDZ (1.5 mg/L) significantly enhanced induction of shoots, maximum shooting frequency, and highest shoot numbers from callus. MS half-strength media with IBA (1mg/L) exhibited early rooting, maximum rooting response, and the highest number of roots. Polyphenols and antioxidant activity in *in vitro* plants were considerably better than in *ex-vitro* plants. Exogenous supplementation of MS solid media with 2,4-D and NAA was the most effective treatment to induce callogenesis, while TDZ was effective for early initiation of shoot formation from callus. The existing protocol has led to increased acclimation and survival of regenerated plantlets with increased polyphenolic quantity and antioxidant activity.

Keywords: Antioxidant activity, Callus induction, Indirect regeneration, Strawberry

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Introduction

Fragaria ananassa (Strawberry) is one of the most economically vital fleshy fruit crops which belong to the Rosaceae family. It is cross-pollinated hybrid soft fruit crop and is monoecious (Guttridge, 2019). Among all other berries, strawberries are commercially cultivated worldwide for having a high percentage of phenolic, flavonoids, Vitamin C, and strong antioxidants (Baby et al., 2018; Farid et al., 2020; Qarni et al., 2022). Strawberry is an aggregate accessory fruit crop and has become unique for its attractive red tone, desirable flavor, sweet aroma, and rich source of fibers, vitamins, and sugar. It is ranked5th in consumption among other berry crops and is popularly consumed either directly or in processed form as milkshakes, pies, juice, jam, chocolates, and frozen yogurt (Jaiswal, 2020). Artificially hybrid strawberry flavors and their sweet aroma are also regularly utilized in items like soap, cleansers, aromatic perfumes, lip shine, and numerous others (Liu et al., 2021). In the 14th century, the first strawberry garden was established by the French. To date, about twenty unique cultivars of strawberries are known and consumed for commercial purposes across the world (Mezzetti et al., 2018). Strawberry is generally cultivated in China, which accounts for 40% delivery of the absolute world contribution, trailed by other leading producers such as the United States, California, Mexico, Turkey, Egypt, and Spain (Hummer & Hancock, 2009; Mezzetti et al., 2018). The climatic factors of Pakistan give an ideal environment for strawberry development. According to recent data, strawberry cultivation in Pakistan is approximately 285-hectare region which is producing 3456 kg ha^{-1} yield (Rajwana et al., 2016; Ali et al., 2021). Strawberry is

a perennial herbaceous long growing, exotic emerging fruit crop and because of genetic transformation advances it can survive in different harsh environments. Strawberry plants are cold resistant and can tolerate temperatures up to –60°C (210F) and produce flowers and fruits at temperatures ranging from 200°C to 290°C (350°F to 850°F). Strawberries grow in little bit acidic soil with the pH ranges from 5.4 to 6.5 (Aghaeifard et al., 2016; Liang et al., 2020).

From a research point of view, it is important to evaluate fruit bioactive profiling for further innovative use as quality control. It is an appreciable source of essential and non-essential nutrients including bioactive antioxidants which have active potential against different types of cancers (Kazmi et al., 2019a; Kazmi et al., 2019b). Strawberry is also enriched with colored pigmented anthocyanin that is associated with a range of health benefits including control of heart problems (Afrin et al., 2016; Khoo et al., 2017) inhibiting tumor development (Lippert et al., 2017) anti-inflammatory potential and scavenging power against oxidative stress (Nardi et al., 2016; Speer et al., 2020). Strawberry stolons or runners are mostly used to commercially cultivate the plant, but this method is complex, time-consuming, laborious, and expensive, and it exposes the plant to the risk of viral disease transmission (Husaini & Neri, 2016; Gaston et al., 2020). However, for vast quantities of dissemination, it is appropriate to use an alternative convenient technique. Plant tissue culture (PTC) is an *in vitro* feasible strategy in which a small section of a plant (explant) is used for vast and rapid production around the year (Kazmi et al., 2019c).

The most concerned and important factor in the micropropagation of plantlets from nodal segments of strawberry explant is the maintenance of hormonal equilibrium. According to numerous studies, plant growth stimulators are preferred for productive callus and other *in vitro* cultures (Ahmad et al., 2012; Uzma et al., 2012; Shah et al., 2013; Ali et al., 2019). Auxin and cytokinin levels were modified to achieve proper callus formation in tissue culture (Khan et al., 2020a). Thidiazuron (TDZ) is recently been used to replace phenyl urea with auxin and cytokinin for shoot initiation in a variety of *in vitro* cultures (Cappelletti et al., 2016). Thidiazuron (TDZ) is broadly utilized as a bioregulator for the expansion of shoots, promotes organogenesis, and enhances numerous developmental activities in plantlets which depend upon explant genotype (Ghosh et al., 2018). The main intent of the experiment was to assess the effect of TDZ on efficient indirect shoot propagation (through callus). Furthermore, the antioxidant ability and level of total phenolic and flavonoid content (TPC, TFC) of *Ex-vitro* and *in vitro* regenerated strawberry plants (*Fragaria ananassa*) were also determined.

Materials and Methods

Seed source, sterilization, and culture establishment

Fragaria ananassa cv. Chandler seeds were obtained fromthe Agriculture Research Institute Tarnab Farm Peshawar. The Seeds were first cleaned with tap water and then distilled water about three times for the removal of dust particles. Then surface sterilization was done by immersing the seeds into a 0.2% (w/v) solution of $HgCl_2$ for two minutes. Residuals of mercuric chloride ($HgCl_2$) solution were removed from the seed's surface by washing with distilled water (DW). The appropriate amount of sucrose (3%) (w/v) and agar (0.8%) (w/v) was augmented to Murashige and Skoog Basal Medium (MS basal media, Sigma Aldrich, catalog number M5519) (Murashige & Skoog, 1962) and before autoclaving pH at 5.8 was maintained by using 0.3 sodium hydroxide (NaOH) or N HCl solutions. Then completely sterilized seeds were inoculated in approximately 10 ml MS basal media containing test tubes. For about three days, the seed cultures were put in a dark place and then transferred to the photoperiod (16:8 h light/dark) where the light was supplied with white cool luminous lamps and adjusted temperature at 25 ± 2 °C and relative humidity with 70%.

Callus induction

In the present experiment, the stem parts about 5-7 mm long were collected from 10-12 days old *in vitro* germinated plants and used as an explant source. The detached stem parts were immersed in MS media that contained different levels (0.5, 1, 1.5, 2 mg/L) of 2,4-Dichlorophenoxyacetic acid (2, 4-D) (Merck, catalog number 820451) individually or with naphthalene acetic acid (NAA) (Merck, catalog number 806862) (0.5, 1 mg/L). A single explant was inoculated in a 10 ml culturing media-containing tube. The data was recorded on days from the frequency of callus initiation and their percentage response. Every treatment culture was kept at room temperature (25 ± 2 °C), followed by an appropriate humidity level (70%) under 16: 8-hour light/dark photoperiod and light at 60 µmol m^{-2} s^{-1} provided to samples from cool white luminous lamps.

Shoot regeneration from callus

To determine the efficiency of Thidiazuron (TDZ) (Thermo Fisher Scientific, catalog number J66663.MD) on the protrusion of shoots from the callus, freshly established calli were sub-cultured on media (MS) comprising various levels of TDZ (0.5, 1, 1.5 mg/L) individually or in combination with Kinetin (0.5, 1, 1.5 mg/L) (Thermo Fisher Scientific, catalog number A13720.03). Data were collected from the days of shoot initiation, shooting incidence, and number of shoots per callus after 5^{th} week of culturing.

Roots induction from regenerated shoots

About 4-5 cm long redeveloped shoots were sub-cultured on fresh established media having half (1/2 MS) and full power of MS basal media that was augmented with diverse amounts of NAA and Indole-3-butyric acid (IBA) (1.0, 1.5 mg/L) either

combined or individually. Data were noted on days to initiation of roots, rooting frequency, and number of roots.

Sample extraction

To scrutinize the antioxidant potential dried powder of *In vitro* and *Exvitro* Strawberry plants were thoroughly assorted with methanol (99.9 %) and vortexed approximately for 5 minutes. Further, the sonication process was followed for half an hour at room temperature (Kazmi et al., 2019c). Finally, the solution was subjected to centrifugation for 10 min at 15,000 rpm, and the supernatant collected was stored at 4 °C for further analysis.

Total phenolic and flavonoid contents

The Total Phenolic Content (TPC) was evaluated using the Folin–Ciocalteu (FC) reagent procedure with slight modification (Kazmi et al., 2019c). In brief, we prepared the reaction mixture by taking a 20 µl methanol extract sample, Folin-Ciocalteu reagent (90 u/L), (Merck, catalog number 109001) and sodium carbonate (90 u/L) to make the mixture volume up to 200 u/L. Gallic acid (1 mg/ml) was applied as a standard and methanol (20 µl) as a negative control. After 90 min, absorbance was measured at 630 nm using a microplate reader; data is documented as Gallic acid equivalent (GAE/g). Likewise, the methodology of Chang et al. was applied with slight changes to measure the total flavonoid content (TFC) (Chang et al., 2002). Briefly, 20 µl samples from each treatment, potassium acetate (10 ul), distilled water (160 ul), and aluminum chloride (10 µl) were taken to achieve 200 uL entire volumes. Quercetin was fixed as the standard or positive control while methanol was a negative control respectively. Data were elaborated as quercetin equivalent per gram of dry weight. Before noting the absorbance at 450 nm by employing a microplate reader, the reaction mixture was incubated for thirty minutes.

DPPH free radical scavenging Activity (%)

To evaluate the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity, the protocol developed by the Abbasi et al. was adopted (Abbasi et al., 2015). In each microplate well, 20 ul of samples were added along with 180 L of DPPH reagent solution (Merck, catalog number 300267), which was then mixed. The microplates were then left in the dark at room temperature for an hour. Different amounts of methanolic samples (10, 5, 2.5, and 1) were considered as negative and (ascorbic acid) as a positive control, added to the 96-well plate. Various ranges such as 190, 195, 197.5, and 199 of DPPH solution (4.8 mg/50 ml) were supplemented with each sample to calibrate the volume to 1000, 750, 500, and 250 g/ml respectively. The absorbance of the solution was detected at 517 nm through a microplate reader.

The below-mentioned formula was used to analyze the DPPH activity.

$$\% \text{ scavenging} = 100 (1 - AE/AD)$$

AE = absorbance of the mixture at 517 nm with the addition of sample

AD = absorbance of DPPH solute without the addition of anything

Statistical analysis

The experimentations were established on a completely randomized experimental design (CRD), and each treatment was conducted thrice. The obtained results were interpreted through One-way statistical analysis of variance (ANOVA) utilizing Statistix 8.1 software. Variations of treatment means were analyzed with the help of the Least Significance Difference (LSD) at a 1% probability level.

Results

***In vitro* seed germinations**

Strawberry seeds were *in vitro* propagated on MS media without the supplementation of any PGRs for a sterile stock explants supply that was used in subsequent studies. After seven days of culture, the highest seed germination level (80%) was observed in the culture tubes.

Determination of appropriate plant growth regulators (PGRs) for callus initiation

To determine a suitable combination of PGRs for callus formation, stem explants obtained from *in vitro* plantlets were inoculated on MS basal media that contained different levels of 2, 4-D separately or along with NAA. Among all the applied media, the earliest callus initiation (7.33 days) and highest callus response (93.33 %) was observed in growth media augmented with NAA (0.5mg/L) + 2, 4- D (0.5mg/L), followed by MS media with 1mg/L of 2, 4-D and NAA (0.5mg/L). While late callus initiation (14.67 days) and lowest callus response was obtained by the explants inoculated on media augmented with 0.5mg/Lof 2,4-D (Table 1).

Table 1 Strawberry Callus induction by augmenting MS media with various levels of 2, 4-D only or in combination with NAA

Media	Levels (mg L^{-1})	Days to callus initiation	Callus induction (%)
Control	0.0	0.00^{e}	0.00^{h}
2,4,-D	0.5	14.67^{a}	53.00^{g}
2,4,-D	1.0	11.33^{bc}	63.33^{de}
2,4,-D	1.5	11.33^{bc}	63.33^{de}
2,4,-D	2.0	10.67^{bc}	60.00^{ef}
2,4,-D	2.5	11.00^{bc}	53.33^{g}
2,4,-D +NAA	0.5 + 0.5	7.33^{d}	93.33^{a}
2,4,-D +NAA	1 + 0.5	9.00^{cd}	83.33^{b}
2,4,-D +NAA	1.5 + 0.5	9.67^{bc}	66.67^{d}
2,4,-D +NAA	2.0 + 0.5	10.33^{bc}	63.33^{de}
2,4,-D +NAA	2.5 + 0.5	11.00^{bc}	63.67^{de}
2,4,-D +NAA	0.5 + 1.0	10.67^{bc}	73.33^{c}
2, 4-D+NAA	1.0 + 1.0	11.67^{bc}	63.33^{df}
2,4,-D +NAA	1.5 + 1.0	12.00^{ab}	60.00^{ef}
2,4,-D +NAA	2.0 + 1.0	11.33^{bc}	56.67^{fg}
2,4,-D +NAA	2.5 + 1.0	12.67^{ab}	53.33^{g}

Value of LSD at $p \leq 0.01 = 1.82$; Mean value with different letters represent significant difference according to 1% probability Level by employing LSD test.

TDZ mediated shoot regeneration and growth parameters

According to mean results, days to shoot initiation from calli masses were significantly influenced by the composition of culture media. It was revealed that those calluses which were cultivated on media containing TDZ+ Kin (1mg/L and 0.5mg/L, respectively) had frequent shoot initiation within 13.33 days, followed by media augmented with 2 mg/L TDZ (16.00 days). While calluses that were cultured on media augmented with 0.5mg/L TDZ took the longest time to initiate shoot induction (28.33 days). Data regarding the rate of shoot initiation and the number of shoot regeneration from callus in media under the response of either TDZ only or in combination with Kinetin showed significant changes among tested media. Table 2 illustrates that in media containing 1.5 mg/L TDZ, the maximum shoot frequency (80%) and amount of shoot generation per callus (5.67) were observed. While in media having TDZ (2 mg/L) showed 71% shoot response and 5.0 shoot developed from callus. The media comprised of TDZ + Kinetin (1mg/L and 2mg/L, respectively), showed the lowest shooting rate (37.33%). Whereas, calluses that were inoculated on MS basal media deprived of PGRs, did not produce shoots.

Table 2 Consequences of different concentrations of TDZ alone or in combination with kinetin on *in vitro* shoots regeneration from Strawberry callus

Media	Levels (mg L^{-1})	Days to shoot initiation	Shoot frequency (%)	Number of developed shoots
Control	0.0	0.00^{h}	0.00^{g}	0.00^{e}
TDZ	0.5	28.33^{a}	49.00^{e}	3.33^{cd}
TDZ	1.0	24.33^{b}	54.00^{de}	3.33^{cd}
TDZ	1.5	21.00^{cd}	80.33^{a}	5.67^{a}
TDZ	2.0	16.00^{f}	71.00^{b}	5.00^{ab}
TDZ + Kn	0.5 + 0.5	19.33^{de}	49.67^{e}	4.33^{bc}
TDZ + Kn	0.5 + 1.5	22.33^{bc}	54.00^{de}	4.67^{ab}
TDZ + Kn	0.5 + 2	18.00^{ef}	54.67^{de}	4.33^{bc}
TDZ + Kn	1 + 0.5	13.33^{g}	56.67^{d}	3.00^{d}
TDZ + Kn	1 + 1.5	17.33^{ef}	56.00^{d}	4.33^{bc}
TDZ + Kn	1 + 2	19.33^{de}	37.33^{f}	3.33^{cd}
TDZ + Kn	1.5 + 0.5	17.67^{ef}	63.00^{c}	4.33^{bc}
TDZ + Kn	1.5 + 1.5	18.00^{ef}	63.33^{c}	3.33^{cd}
TDZ + Kn	1.5 + 2	17.33^{ef}	53.67^{de}	4.33^{bc}

Mean value with different letters represent significant difference according to 1 % probability; Level by employing LSD test.
Value of LSD at $p \leq 0.01 = 1.82$

Roots formation and acclimatization

The present experiment showed that the rooting response of callus was more frequent on half-power MS media compared to full-strength MS media. Furthermore, rapid rooting was observed in cultures comprising IBA as compared to NAA-augmented media. The minimum days to root formation (8.67 days), maximum root induction frequency (66.33%), and higher number of root plantlets (6.33) were recorded in shoots cultured on half strength of MS media augmented with IBA (1mg/L). While, late root initiations (15.33 days), Poor rooting response (24.33%), and a minimal number of roots (2.00) were recorded in shoots that were cultured on full MS basal media without any PGR (Table 3). The healthy *in vitro* strawberry plantlets were also uniformly relocated to the vermin culture and positioned in the growth chamber until primary hardening was achieved. Following the successful acclimatization of the plants under primary hardening, all the plants were moved to the field (natural environment) after three weeks. 70% of the plants survived with significant growth rates (Fig. 1).

Table 3 Root induction from callus derived shoots in response to Full and Half strength of MS media augmented with different levels of NAA and IBA

Media	Levels (mg L^{-1})	Days to roots initiations	Rooting %	Average number of roots
MS media	Full	15.33^{a}	24.33^{f}	2.00^{e}
MS media	Half	12.67^{b}	28.00^{f}	2.67^{de}
MS + IBA	Full + 1.0	12.33^{b}	39.00^{d}	3.00^{cde}
MS + IBA	Full + 1.5	10.67^{c}	56.33^{b}	3.67^{bcd}
MS + NAA	Full + 1.0	13.33^{b}	34.00^{e}	3.00^{cde}
MS + NAA	Full + 1.5	13.67^{b}	46.33^{c}	4.00^{bc}
MS + IBA	Half +1.0	8.67^{d}	66.33^{a}	6.33^{a}
MS + IBA	Half + 1.5	9.33^{cd}	56.67^{b}	4.67^{b}
MS + NAA	Half + 1.0	10.33^{c}	42.33^{c}	3.67^{bcd}
MS + NAA	Half + 1.5	12.67^{b}	59.00^{d}	4.06^{bc}

Mean values with different letters represent significant difference according to 1% probability Level by employing LSD test.
Value of LSD at $p \leq 0.01 = 6.05$

Evaluation of phytochemical and antioxidant potential in strawberry plant

Total phenolic contents, total flavonoid contents, and antioxidant capacity of extracts from *ex-vitro* and *in vitro*-produced strawberry plants were investigated in this study. There were major variations in polyphenol synthesis between *in vitro* and *ex-vitro*-grown plants, according to the findings. Maximum polyphenols contents and antioxidant activity (TPC= 2.8, TFC= 1.8, and DPPH= 84%) were detected in *in vitro* plants. However, a moderate level of phytochemicals and antioxidant activity (TPC= 2.3, TFC= 0.5, and DPPH= 70%) was detected in the *Ex-vitro* plant (Fig. 2).

Discussion

The primary goal of the research conduction was to provide a rapid and effective protocol for *in vitro* regeneration of strawberry plants from callus with significant antioxidant potential. In this research work, the maximum seeds germination frequency (80%) of Strawberries was obtained from MS media without any addition of PGRs for a sterile stock explants supply that was further used in subsequent experiments. *In vitro* seed propagation has shown to be an efficient method for delivering disinfected germplasm as a font of explants, and it can naturally decrease infectivity risks. During plant tissue culture practices, explants isolated from *in vitro* generated plantlets are generally free of microbial contamination infection than the explants obtained from field germinated plants (Khan et al., 2020a).

The exogenous application of PGRs in the culture media is an essential step in inducing diversity in explant capacities and the development of *in vitro* callus culture (Satish et al., 2015; Shah et al., 2015; Jan et al., 2015; Shah et al., 2020; Ahmad et al., 2020). To determine the efficient PGR combination for callus initiation, stem explants from *in vitro* plantlets were inoculated on MS medium that contained different concentrations of NAA and 2, 4-D separately or in combinations. NAA and 2, 4-D are two plant growth regulators that are usually exploited in plant tissue culture for callus induction (Akter et al., 2008; Abd Elaleem et al., 2015). In the current research, a mixture of 2, 4-D, and NAA was more efficient at lower concentrations for rapid initiation and high induction rate of callus culture from stem sections of strawberry, as compared to alone. The growth medium supplemented with NAA (0.5 mg/L) + 2, 4-D (0.5 mg/L) had the earliest callus commencement (7.33 days) and most significant callus response (93.33%). Explants placed on medium supplemented with 0.5 mg/L of 2, 4-D had the lowest callus response and delayed callus initiation (14.67 days) (Table 1). Previous research reported that a low dose of 2,4-D combined with NAA resulted in callus initiation earlier in *Cucurbita pepo* (Pal et al., 2007) and *Solanum melongena* (Qin et al., 2017).

Auxin promotes cell division, cell elongation, and cell formation, which is essential for plant growth, development,

and morphogenesis (Mohammad et al., 2019). The reduction in callus growth was observed when exposed to higher levels of 2,4-D in the culture media. Auxins like 2,4-D may have herbicidal property at high concentrations, produce toxicity, inhibits the formation of callus, and cause browning in callus cells (Sundram et al., 2012). Comparable to our consequences, the combinatorial impact of 2,4- D and NAA favored the callus growth in many other plants such as *Lemna minor* (Kaviani, 2014) and *Caralluma tuberculata* (Zafra- Stone et al., 2007).The content of the cultural media has a significant impact on callus regeneration capacity. Kinetin is a form of cytokinin explored in the regeneration and multiplication of shoots (Anisuzzaman et al., 2010; Narendran et al., 2013). Currently, bioregulator TDZ is frequently utilized to enhance shoot initiation and proliferation in several plant species (Faisal et al., 2014), and it can substitute the utilization of many other cytokinins (BA, and BAP). In the present study, a low concentration of TDZ with Kinetin led to rapid regeneration of plantlets from callus. A similar impact of TDZ potential in shoot regeneration had been reported in many other plant species such as *Exacum travancoricum* (Kannan et al., 2007) and *Mentha arvensis* (Faisal et al., 2014).

Data regarding shooting frequency and number of shoot development from callus in response to TDZ separate and combination with Kinetin demonstrated significant variation in tested media. In the current experiment kinetin and TDZ in association were not substantially more productive than TDZ alone for numerous shoot proliferations. Metabolic cascade induced by TDZ results in the alteration of major signaling pathways. For instance, it shifts auxin endogenously and acts as a signaling molecule which in turn stimulates morphogenetic potential (Biesaga-Kościelniak et al., 2010). Kinetin has commonly used cytokinin in *in vitro* culture media for shoot regeneration and is considered a strong beneficial agent to promote shooting in plants (Guo et al., 2011). It was observed that calluses inoculated on TDZ+ Kin medium (1 mg/L and 0.5 mg/L, respectively) frequently initiated shoots in 13.33 days, whereas media supplemented with 2 mg/L TDZ took 16.00 days. While calluses planted on medium containing 0.5 mg/L TDZ took the maximum days (28.33 days) to start the shoot induction process. The highest shoot frequency (80%) and amount of shoot generation per callus (5.67) were recorded in the medium containing 1.5 mg/L TDZ (Table 2). The findings of the current experiment are contrary to the findings of Jain and Ochatt, (Jain & Ochatt, 2010) who mentioned that more shoots from callus can be obtained by augmenting growth media with the collaborative impact of TDZ and kinetin as compared to TDZ only.

The major barrier to the effectiveness of plant tissue culture is weak *in vitro* rooting (Dewir et al., 2016; Oakes et al., 2016). In current findings, half-power MS media had a faster rooting response than full-strength MS basal media. Furthermore, as opposed to NAA, a rooting medium augmented with IBA resulted in rapid root induction. According to Barik et al.(Barik et al., 2004) nutrient insufficiencies serve as strong stimulants for rhizogenesis. It has been recognized that half-power media with low doses of salts can be more operative for *in vitro* root induction, because of the low nitrogen contents and suitable osmotic potential (Barik et al., 2004). Similarly, healthy root culture produced on MS medium with half strength and augmented with IBA has been documented in numerous plants such as *Ficus anastasia*, *Jatropha curcas* (Rathore et al., 2015), and *Pogostemon cablin* Benth (Swamy et al., 2014). Moreover, in our results, the supplementation of IBA (1 mg/L) tremendously improved root development. In shoots cultivated on half strength MS medium supplemented with IBA (1 mg/L), the lowest days to root formation (8.67 days), highest root induction frequency (66.33%), and highest number of root plantlets (6.33) were observed (Table 3). Higher concentration of IBA in growth media drastically reduced root induction. Several levels of IBA were more influential for early root development as compared to NAA-supplemented media. It can be concluded that the employment of IBA at 1.0 mg/L enhanced the root formation in strawberries.

Many plant species contain polyphenols, which are a vital factor in managing plant growth and production. These metabolites have an array of roles in plants, including phytoalexin-mediated protection against microbial attack, enzyme modulation, lignification, auxin activation, and cell wall development (Kazmi et al., 2019d; Shah et al., 2020; Anum et al., 2021; Mahmood-ur-Rehman et al., 2021; Shereen et al., 2021; Ehsan et al., 2022). *In vitro* shoot cultures for plantlet development are a promising approach for producing secondary metabolites, particularly when the metabolites are produced in feasible amounts (Amoo et al., 2012; Khan et al., 2020b). In the current research investigation, the total phenolic and flavonoid contents, and total antioxidant potential of extracts obtained from *Ex-vitro* and *in vitro* germinated plants of strawberries were evaluated. The metabolic synthesis of *ex-vitro* and *in vitro*-raised strawberry plants differed significantly. The overall quantity of phenols and flavonoids in the extracts of *in vitro* cultures was higher as compared to *ex-vitro* generated plants, according to quantitative examination. The maximum quantity of TPC (2.8 mg) and TFC (1.2 mg) was recorded *in vitro* strawberry plants as compared to *Ex-vitro*.

In a previous report, the metabolites in *in vitro* propagated *M. plumber* were manipulated by the presence of cytokinin (TDZ) in culture media and most of the phenolic acids were enhanced in *in vitro* germinated plants than the *Ex-vitro* plantlets (Aremu et al., 2013). The influence of PGR especially cytokinin in a culture medium is essential for the production of bioactive metabolites that are capable of scavenging free radicals (Kazmi et al., 2019a). In the present findings, maximum DPPH (84 %) were detected in *in vitro* plants. From the current results, we recommend that utilization of the *in vitro*-derived shoots through indirect organogenesis might be feasible for the accumulation of biologically active compounds with significant antioxidant activity in strawberry plants.

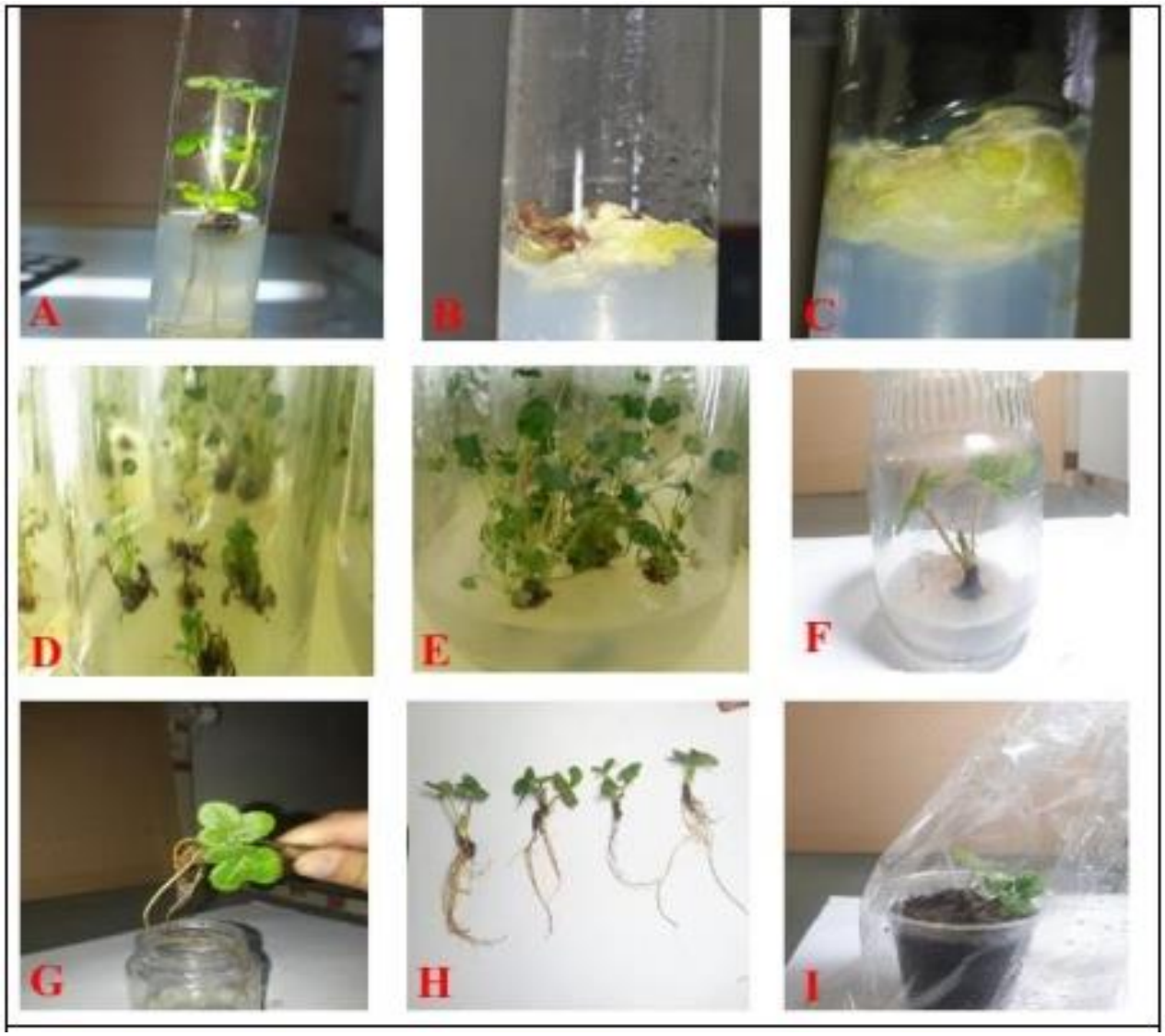

Fig. 1 Indirect organogenesis of strawberry. *In vitro* germinated plant (A); Callus initiation from stem explants (B); Callus induction (C); Shoot initiation from callus (D); Multiple shoot induction from callus (E); Root induction from regenerated shoot (F); *In vitro* rooted plants (G, H); Hardening and acclimation of *in vitro* regenerated plant (I).

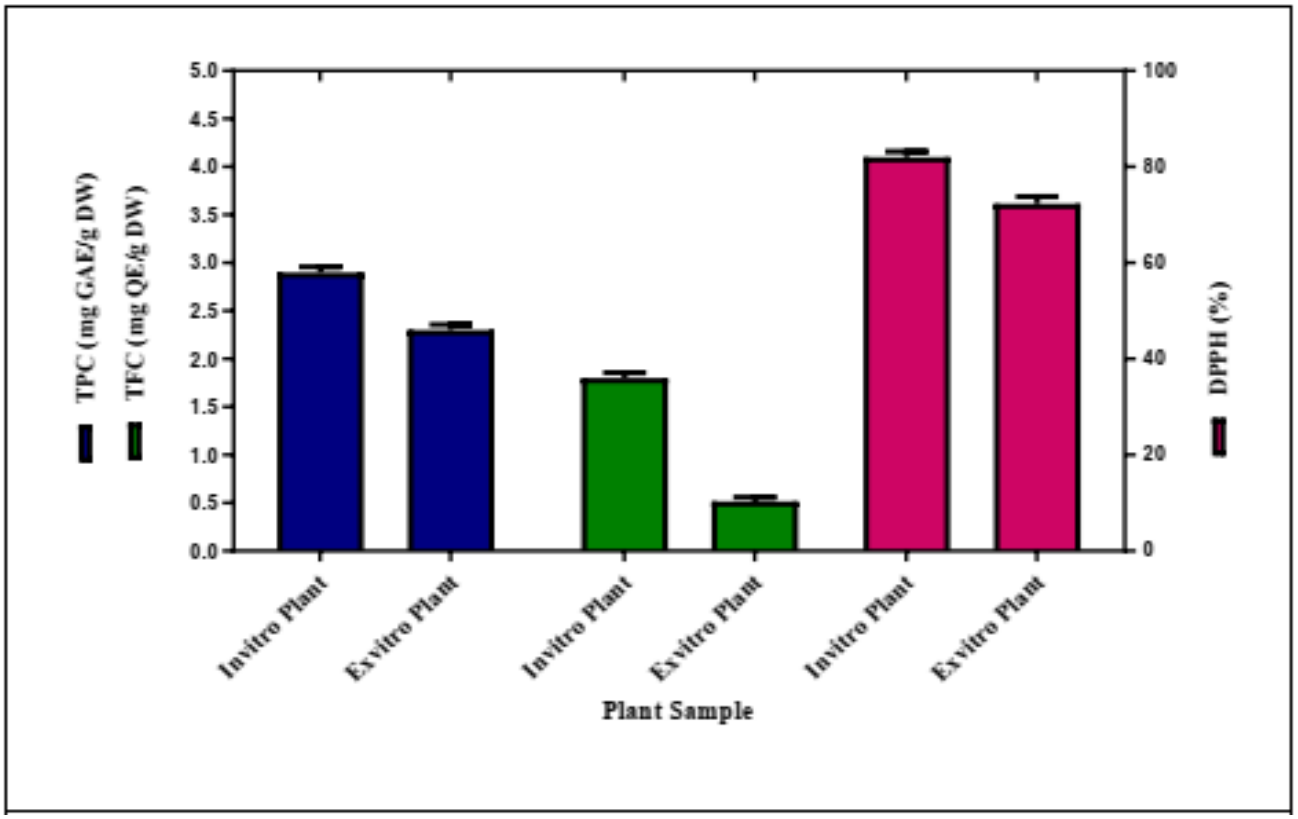

Fig. 2 Evaluation of total phenolic and flavonoid contents, and total antioxidant capability of extract obtained from *in vitro* and *Ex-vitro* germinated plants of *F. anannsa*

Conclusion

In current research work, we developed a highly efficient plant regeneration protocol by using a callus, derived from the stem (explant) of an *in vitro* germinated plantlet of *Fragaria annansa*. Our experiment concluded that exogenously supplemented MS solid media with 2,4-D and NAA (0.5mg/L + 0.5mg/L) is optimum to prompt maximum percent response of callus induction. TDZ resulted to be significantly proficient for initiation of shoot formation from callus, while IBA enhanced root growth, which resulted in improved regeneration, environmental adaptation, and survival of plantlets. The treatment of callus with TDZ and IBA significantly enhanced the polyphenol content (TPC, TFC) and total antioxidant capacity (DPPH) in *in vitro* germinated plants than *ex-vitro* plants. This protocol can be utilized for the commercial scale production of strawberries (*Fragaria ananassa* Cv. Chandler) with enhanced metabolic profile and antioxidant activity.

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