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Micropropagation and Callus Induction in Pureline Cultivars of Tomato (Solanum Lycopersicum L.)

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Abstract

Tomato (Solanum lycopersicum L.) is a significant vegetable crop as it has both nutritional and economic importance on a global scale. Tomato breeding programs must be able to multiply and improve genetically and through biotechnology through efficient systems of in vitro regeneration. The present study aimed to streamline micropropagation and callus induction procedures with two pure-line tomatoes (BL-1174 and Tinto) with varying auxins and cytokinin combinations. The study involved two experiments that were designed using a completely randomized design (CRD): one was aimed at testing the regeneration of shoots and roots in an experiment using nodal explants, and the other aimed at callus induction with leaf disc explants. Regeneration was undertaken in Murashige and Skoog (MS) medium supplemented with different concentration levels of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA), whereas 2,4-D and BAP were used in callogenesis. Analysis of variance (ANOVA) was used in data analysis, and the means of treatment were compared with the help of the Tukey HSD test. Treatments and genotypes had significant differences in all the parameters that were evaluated. Tinto pure line had better in vitro performance with the highest induction of shoot (74.3%), shoot length (2.7 cm), root induction (71.7%), number of leaves and roots per explant, survival percentage (68%), and callus induction (90) with increased concentration of BAP and IBA. Internodal distance was found to be relatively higher in BL-1174, and the total regeneration efficiency was lower. The modified hormonal ratios exhibited high genotype responses, as Tinto was more responsive to in vitro regeneration and formation of callus. The protocol developed is a reliable and reproducible system to consider large-scale micropropagation and as a basis for genetic transformation, mutation breeding, and other high biological applications of tomato.

Keywords: America, tomato, nutrient, Antioxidants, beta-carotene, fibre, lutein, lycopene, vitamins, regeneration.

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1. Introduction

Pakistan is an agricultural nation, with the agricultural sector being a major source of raw material to major industries and a source of employment to a large percentage of the population. In 2021, the gross domestic product (GDP) of the country was about 16% of agriculture that included forestry, livestock, and fisheries (FAO, 2023). This industry contributes significantly towards food security, poverty relief, industrial growth, and the provision of jobs to approximately 44 percent of the urban and 62 percent of the rural population. Pakistan has an area of 796,095 km 2, of which almost 22 million hectares are under cultivation, and 8.3 million hectares are non-cultivated. Major crops make up 6.5 percent of the GDP, whilst minor crops, forestry and fisheries make up 2.3 percent, 0.2 percent, and 0.4 percent, respectively (Azam and Shafique, 2017). Food insecurity is experienced by over 48 percent of the population, regardless of the rising food production, partly because of the rapid growth of the population, overuse of agricultural water resources and the calamities like climate change that are still putting pressure on the limited water resources.

Tomato (Solanum lycopersicum L.) is a vegetable crop that is extensively grown and eaten all over the globe and falls in the family Solanaceae. It possesses a short life cycle, well-characterized genetic resources and a diploid genome (2n = 24), which makes it a perfect model crop in in vitro and molecular investigations (Chaudhry et al., 2010). Tomato is a healthy nutrient, as it is a valuable source of vitamins (B and C), minerals (Fe and P), amino acids, sugars, and dietary fibre. It is also a significant source of dietary lycopene, a powerful antioxidant that is linked with a decrease in cardiovascular diseases and cancer (Guil-Guerrero and Rebolloso-Fuentes, 2009). The tomato fruits are widely used as fresh and processed products in the form of sauces, soups and juices (Dam et al., 2005). Tomato has emerged as an important crop in the world due to its nutritional value and the growing demand for the product among consumers (Chaudhry et al., 2019).

Tomato has key micronutrients (Fe, Mn, Zn, and Cu), as well as macronutrients (K, P, Mg, and Ca), nutraceutical compounds, including potassium, lycopene, folate, vitamin C, and 1,4-carotene, which have a beneficial effect on human health (Durrani et al., 2021). Tomato is a climacteric fruit, and this causes it to become ripe very quickly. High respiration rates, microbial infection, and a poor shelf life can lead to huge losses after harvest (Salam et al., 2023). As a result, enhancing the firmness, colour, nutritional quality and shelf life of fruits has still been of significant concern in tomato breeding efforts and research (Handa et al., 2017).

Tomato is the most produced vegetable crop globally, with over 170 nations producing tomatoes, and the production is about 186 million tons in 2022 (FAO, 2023). Today, there was an overall production of 189.1 million tons of tomatoes in the world, with China producing the largest volume (34.72 million tons) and India (20.57 million tons). Mean world has 36.97 t/ha, and total production is expected to be 51.93 million tons in 2026 (Durrani et al., 2021).

Tomato production in Pakistan is 574, 052 tons on an average area of 58, 196 hectares, 36 th in the world. The major tomato producing province is Balochistan, then Punjab, Khyber Pakhtunkhwa (KPK), and Sindh. The variety of agro-ecological regions of Pakistan makes the production of tomatoes possible throughout the year. Nevertheless, tomato farming has several

limitations that are encompassed by the expensive nature of disease-resistant varieties, fertilizers and herbicides, lack of knowledge on disease-resistant varieties and poor fertilizer application (Jat et al., 2012). Also, abiotic stress, which includes extreme temperatures, humidity, excessive rainfall, water stress, and low light intensity, and biotic stresses such as pests, diseases, and poor soil conditions, have devastating impacts on tomato productivity (Hasanuzzaman et al., 2020). Since tomato is a sensitive crop to environmental changes, the molecular and physiological processes of environmental tolerance to stress can be used as supportive instruments to a successful breeding program (Gerszberg and Hnatuszko-Konka, 2017).

Plant tissue culture has become an effective and dependable method of multiplication of the tomato vegetable of high quality and disease-free on a large scale. It involves the growth of plant cells, tissues or organs in vitro under controlled and aseptic conditions with the help of nutrient medium (Thorpe, 2007). Tissue culture methods are used to micropropagate, induce callus, induce somatic embryogenesis, culture anthers and protoplasts, and are useful in genetic transformation methods, such as the Agrobacterium-mediated and biolistic techniques (Samantararay et al., 2024). These methods increase the breeding schedules, limit the seasonal restrictions, and allow the rapid multiplication of the elite genotypes (Idowu et al., 2009).

The regeneration of tomatoes has been reported to be accomplished utilizing different types of explants (long-leaf, nodal parts, roots, and shoot apices) with the help of various auxins and cytokinins (George et al., 2008; Chaudhry et al., 2010). The regeneration responses, however, differ significantly among cultivars, and it is challenging to find a universal protocol that can be made (Ahmad et al., 2011). It has been reported that optimization of plant growth regulator (PGR) combinations in Murashige and Skoog (MS) medium had improved callus induction and efficiency of the process in the regeneration of shoots (Hussain et al., 2013). Tissue culture is also critical in regeneration, besides generating disease-free plants and resistance to biotic and abiotic stresses using genetic modification.

The current experiment aimed to germinate and multiply pure-line tomato seeds in controlled in vitro conditions and to determine how various doses of growth regulators of plants can influence growth, regeneration and growth development of tomatoes. It is hoped that the findings will be used in the future to develop a reproducible and efficient micropropagation of tomatoes in vitro protocol that has practical uses in commercial micropropagation and tomato improvement programs.

2. Materials and Methods

The experiment was carried out in the Plant Tissue Culture Lab, Department of Horticultural Sciences, University of Agriculture, Faisalabad. The study was divided into two major experiments:

1. In vitro Regeneration of tomato.
2. Callus Induction.

2.1 Media Preparation

The basic medium on which tomato (Solanum lycopersicum L.) was to proliferate, culture, and regenerate was Murashige and Skoog (1962) salt mixture with various levels of concentrations of BAP and IBA added. Stock solutions of growth regulators and main salts were made using distilled water. Growth regulators and salts were introduced into the medium, and the pH was altered to 5.6.

The carbon source (sucrose with 30 g/L) and solidifying agent (agar with 8 g/L) were added. Media were transferred into test tubes (10 mL each tube) and capped with plastic caps and sterilized in an autoclave at 121 o C and 15 psi for 20 minutes. In vitro Regeneration of Tomato 2.2 Experiment 1: In vitro Regeneration of Tomato.

2.2 Experiment 1: In vitro Regeneration of Tomato

2.2.1 Explant Sources

The seeds of tomato (BL-1174 and Tinto) were pure-line seeds that were obtained at the Plant Breeding and Genetics Department, University of Agriculture, Faisalabad.

2.3 Explant Sterilization

Seed surface sterilization was done by placing the seed in 70% ethanol (v/v) with one or two drops of Tween-20, after which it had to be rinsed three times in sterile double-distilled water. The seeds were then immersed in 5% sodium hypochlorite (v/v) solution for 2 -3 minutes, and 3 rinses in sterile distilled water were done. The sterilized seeds were grown in modified MS medium.

2.4 Seed Culture

Seeds were germinated on MS basal medium (Murashige and Skoog, 1962) under controlled in vitro conditions.

2.5 Micropropagation of In Vitro Seedlings

In vitro-grown seedlings were prepared into nodal explants that were then cultured using MS medium, based on various levels of BAP and IBA to induce shoot development.

2.6 Media Composition

The explant was then placed in complete MS medium with the plant growth regulators (PGRs) in different concentrations to encourage shoots to develop.

Treatments: MS + BAP + IBA

Table 3. 1 Media induction of shoot among explants.

Treatments	BAP (mgL^{-1})	IBA (mgL^{-1})
T0	0	0
T_1	1.0	0.5
T_2	1.5	1
T_3	2.0	1.5
T4	2.5	2.0
T5	3.0	2.5

2.7 Sterilization of Media

All media were sterilized as described above and incubated at 25 ± 2°C under a light intensity of 2500 lux.

2.8 Root and Shoot Regeneration

Nodal explants were observed for root and shoot regeneration on modified MS medium. Parameters recorded included:

1. Days to shoot induction
2. Shoot length (cm)
3. Number of shoots per explant
4. Shoot induction percentage (%)
5. Number of roots per explant
6. Root length (cm)
7. Number of leaves
8. Plant height (cm)
9. Root induction percentage (%)
10. Internodal distance (cm)
11. Plant survival percentage (%)

Measurements were taken using a standard measuring scale, and percentages were calculated using appropriate formulas.

2.9 Experimental Layout (Experiment 1)

The experiment was arranged as a Completely Randomized Design (CRD) in factorial arrangement with six treatments and two cultivars. Each treatment had three replications, and standard statistical procedures were used for analysis (Steel et al., 1997).

2.10 Experiment 2: Callus Induction

2.10.1 Explant Sources

Leaf discs from in vitro-grown plantlets of BL-1174 and Tinto were used for callus induction.

2.10.2 Media Composition

Callus induction and subculture were performed on MS basal medium supplemented with different concentrations of 2,4-D and BAP:

Table 3.2 Media preparation of callus induction in callus.

Treatments	MS media + PGRs (2-4D+BAP) (mgL^{-1})
To	0
T1	1+0
T2	2+1
T3	3+1.5
T4	4+2
T5	5+2.5

2.11 Data Collection (Experiment 2)

1. Days to callus induction: Recorded after culturing explants on callus induction medium.
2. Callus induction percentage (%):

$$\text{Callus induction \%} = \frac{\text{no. of callus induction}}{\text{total no. of callus culture}} \times 100$$

Calculated after 4-5 weeks of culture.

2.12 Experimental Layout (Experiment 2)

The callus induction experiment was conducted as a CRD in factorial arrangement with four treatments, two genotypes, and three replications. Data were analyzed using standard statistical methods (Steel et al., 1997).

3. Results and Discussion

Experiment No. 1

3.1 In vitro regeneration of tomato

Aspirations and cultural approaches of the tomato have been modified to regenerate tomatoes under in vitro conditions. In the current experiment, tomato was suitable in regeneration systems using the tissue culture-based system by demonstrating effective regeneration of the plant using optimized plant growth regulator (PGR) combinations. Regeneration response was not consistent across pure lines, so there was a significant effect of genotype on in vitro performance.

3.1.1 Time spent to shoot induction

The analysis of variance showed that there was a highly significant impact of in vitro regeneration of tomato pure lines BL-1174 and Tinto when plant growth regulators (PGRs) were used, and the interaction between the genotypes and the treatments had a significant impact ($p = 0.0338$). The findings show that genotype and hormonal treatment are important to promote shoot induction. The pure lines and PGRs were compared, and it was found that the number of days needed to induce shoots was reduced with the increase in the concentration of PGR. The Pure line 2 (Tinto) caused shoots to induce quicker than Pure line 1 (BL-1174). Treatment T6 recorded the lowest days required to shoot induction, i.e. 19 days in Pure line 2 (Tinto) and 22 days in Pure line 1 (BL-1174). On the other hand, Figure 1 indicates that the highest number of days was taken in the control treatments, i.e., 26 days in Pure line 2 (Tinto) and 27 days in Pure line 1 (BL-1174).

These results indicate that the addition of MS medium with 3 mg/L BAP and 2.5 mg/L IBA has a great positive impact on the initiation of shoots when the hormones are used, and the auxins fail to promote the shoot formation in the absence of the hormones. The improvement in the shoot induction of Tinto over BL-1174 may also be because of the differing genetic composition of the two pure lines that may have an effect on the responsiveness of the lines to the PGRs.

The findings can be related to the research conducted by Rashid and Bal (2010), who also found the decreasing induction time of shoots when the same level of BAP and IBA was used. Also, the current findings indicate that the most

important consideration of PGR concentration is required to enhance the efficiency of micropropagation protocols in tomato, which is congruent with the overall inquiry into Solanaceae tissue culture research.

Table 1, Annexure (A)

3.1.2 Number of shoots per explant

The number of shoots per explant increased significantly with increased concentration of BAP and IBA in micropropagation of tomato pure lines, BL-1174 and Tinto. The concentration of cytokinin and auxin increased and stimulated the shoot proliferation, proving the positive synergistic action of BAP and IBA in promoting shoot proliferation. Pure lines 1 (BL-1174) and 2 (Tinto) proved to be responsive, with Tinto always giving more shoots. The analysis of variance showed that the effects of PGRs and pure lines on the number of shoots were very significant, and also the interaction of the genotype and treatment was found to be statistically significant. PGR treatments were compared, and the highest shoots per explant (3.6667) were obtained by T6 (3 mg/L BAP + 2.5 mg/L IBA) in Pure line 2 (Tinto), and T5 obtained the highest number of shoots (2.1667) in BL-1174. On the other hand, the control and T2 treatments registered the lowest number of shoots, with the mean values of the sample being about 1.3333 in BL-1174 and 1.1667 in Tinto, respectively (Figure 2).

These findings indicate that the auxins and cytokinins induce a complementary effect in shoot induction, whereby cytokinins induce the formation of a shoot bud, and auxins induce cell division and elongation. The given direction correlates with the promotive influence of auxins on the number of shoots mentioned by Deklerk et al. (1999). Likewise, Elfil et al. (2023) affirmed the augmenting nature of concomitant PGRs interventions in tomato micropropagation. The current results also concur with Ishag et al. (2009), which states that the optimal concentration of BAP and IBA is paramount in attaining maximum multiplication in tomato tissue culture. The increased shoot growth in Tinto over BL-1174 could be a result of the genetic differences inherent in the sensitivity of cytokinins and meristematic activity.

Table 2, Annexure (B)

3.1.3 Shoot length

Micropropagation was done on tomato pure lines BL-1174 and Tinto, and it was revealed that as the levels of BAP and IBA increased, the length of the shoots grew dramatically. Shoot length and concentrations of PGR were found to have a positive correlation, which showed that cytokinins and auxins work together to enhance cell elongation and shoot growth. The analysis of variance revealed that the treatment (P 0.01), but not the interaction effect (P 0.01), affected the shoot length, and that the genotypes (P 0.05) also had a significant effect. Higher concentrations of PGR had a strong promoting effect on shoot length, with the highest result being achieved under T6 (3 mg/L BAP + 2.5 mg/L IBA). Pure line 2 (Tinto) had longer shoots than Pure line 1 (BL-1174), as it had outperformed in other morphogenic characteristics. On the other hand, the shortest shoot length was found in the control treatments, 2.1833 cm in the case of BL-1174 and 2.2167 cm in the case of Tinto (Figure 3).

These findings suggest that cytokinins such as BAP increase the apical meristem activity in the shoot and induce cell division, whereas auxins such as IBA promote cell elongation that in turn results in the formation of longer shoots. The inclusion of MS medium with 3 mg/L BAP and 2.5 mg/L IBA, therefore, largely

enhanced the shoot elongation rate relative to the hormone-devoid control. These results correspond with the previous research by Deklerk et al. (1999), Elfil et al. (2023), and Ishag et al. (2009), who found the facilitated shoot development of tomato tissues by the optimum cytokinin-auxin ratios. It is also possible that the enhanced shoot growth in Tinto was owing to genotype-specific sensitivity to cytokinins and enhanced meristematic activity, and it is emphasized that genotype selection is vital in in vitro propagation procedures.

Table 3, Annexure (C)

3.1.4. Shoot Induction Percentage

Micropropagation of tomato pure lines BL-1174 and Tinto showed that a high concentration of BAP and IBA had a significant effect on a high percentage of shoot induction. Analysis of variance revealed that PGRs, as well as the genotypes, had a very significant effect on the percentage of shoot induction, but their interaction was not found to be statistically significant. Treatments and pure lines were compared, and the maximum level of shoot induction was observed in T6 (3mg/L BAP and 2.5mg/L IBA), whereby BL-1174 and Tinto gave 53.33 per cent and 74.33 per cent, respectively. Conversely, the control treatment had the lowest percentages of induction, 23.33% BL-1174 and Tinto (Figure 4).

These results underscore the fact that the optimized cytokinin-auxin in the case of nodal explants can lead to the initiation of the shoot, which is in line with the classical model that auxin-cytokinin balance regulates the initiation of organogenesis. This increased shoot induction in Tinto can be explained by genotype-specific sensitivity to cytokinins, which increases meristematic activity and organogenic ability. The findings indicate that IBA (2.5mg/l) and BAP (3mg/l) supplemented into the MS media significantly enhance shoot induction in comparison to the control media with no hormones. This is consistent with previous data by Jehan and Hassanein (2013) that the ratio of auxin to cytokinin is crucial to trigger the shoots in tomato tissues and optimize the regeneration rate.

3.1.5 Physiological Mechanisms Underlying Hormonal Responses

Physiology behind Hormonal Responses. The varying sensitivity of tomato pure lines (BL-1174 and Tinto) to different levels of BAP and IBA could be attributed to the fact that the well-known physiological functions of cytokinins and auxins in morphogenesis in

Table 4, Annexure (D)

plants have been established. The cytokinins, e.g., BAP, are the ones that mainly enhance cell division through activation of cyclin-dependent kinases, promote shoot meristem activity, enhance chloroplast differentiation, and inhibit senescence, thus facilitating the initiation and multiplication of shoots. The auxins, such as IBA, also control the cell elongation, the development of the vascular tissue, and the development of adventitious roots by polar auxin movement, and the control of the gene expression related to the meristem differentiation (George et al., 2008).

In this experiment, the increased cytokinin to auxin ratio that was recorded in T6 was conducive to shoot induction and multiplication, which accounts for the increased number of shoots, shoot length, and percentage shoot induction over control treatments. On the other hand, the comparatively higher concentrations of auxin were more useful in stimulating root growth and in inducing callus, as observed during the earlier root development stages.

The combined effects of BAP and IBA were especially critical in the regulation of organogenesis because the cytokinin-stimulated shoot proliferation and auxin-stimulated root initiation had synergistic effects on the morphogenic response. These observations are in line with the theory of classical hormonal balance and previous observations done in tomato and other Solanaceous crops (George et al., 2008; Jehan and Hassanein, 2013).

In addition, genotype-specific variations, including the increased shoot response of Tinto relative to BL-1174, indicate that an endogenous level of hormones and receptor sensitivity also play a role in the observed alternative in morphogenic potential.

3.1.6 Days Taken to Root Induction

Micropropagation of tomato pure lines (BL-1174 and Tinto) showed that the period of root induction declined slowly with the increase in concentrations of BAP and IBA. According to this trend, the high concentration of PGR promotes the rapid initiation of root development by increasing cell division and differentiation of the root primordia. Interestingly, in spite of the fact that higher levels of IBA and BAP favoured the elongation of roots, high levels of cytokinins are generally known to retard root elongation, which could be the cause of the genotype-specific differences. The analysis of variance showed that PGRs and the pure lines were significantly higher in the number of days it takes to induce roots; however, there was no statistical significance between the two factors. Treatment comparison also revealed that the highest number of days required to induce roots was in the control, using treatment of 46.33 and 46.167 days in BL-1174 and Tinto, respectively. Conversely, the fewest number of days was in treatment T6 (3 mg/L BAP + 2.5 mg/L IBA), which took 41.50 days in BL-1174 and 40.50 days in Tinto (Figure 6).

The decrease in the time of root induction in T6 is probably associated with the synergistic action of BAP and IBA, in which cytokinins induce early meristem growth, and auxins induce cell enlargement and vascular differentiation. These findings are compatible with the results of Rashid and Bal (2010), who indicated that concomitant application of auxins and cytokinins is effective in increasing the growth of tomato roots in the process of micropropagation. Also, the Tinto pure line took around the same span as BL-1174, with about one more day, demonstrating genotypic differences in the responsiveness to PGRs, possibly due to differences in the level of endogenous hormones and tissue sensitivity.

Table 5, Annexure (E)

3.1.7. Root Length

Micropropagation of tomato pure lines BL-1174 and Tinto revealed that root length increased tremendously with the increasing concentration of BAP and IBA. The analysis of variance revealed that PGRs, pure lines, and their interaction significantly affected the root length and ascertained that the genotype treatment and hormone treatments are essential in the development of roots. Treatment comparison displayed that at T4 (2.0 mg/L BAP + 1.5mg/L IBA), the maximum root length was noted in BL-1174 and Tinto, and was 0.9 cm and 1.15cm, respectively. However, the shortest root length was found in the control treatments and was 0.166 cm in BL-1174 and 0.616 cm in Tinto (Figure 6). This increase in root length at higher levels of PGR has been explained by the fact that the auxin (IBA) promotes cell elongation and vascular differentiation, whereas cytokinins (BAP) indirectly promote root development by promoting

shoot-derived signals through which root development is regulated.

These results are consistent with the results of Deklerk et al. (1999), who have reported that auxins stimulate root induction and elongation of tomato explants. On the same note, Elfil et al. (2023) established that a combination of optimal auxin and cytotoxin leads to a vegetative increase in roots. It was interesting to note that the Tinto pure line always had longer roots compared to BL-1174, indicating genotypic variations in sensitivity of PGRs, which might be because of the difference in endogenous auxin concentration and tissue sensitivity. Ishag et al. (2009) also recorded these same results, as they found that there were differences in root elongation in tomato genotypes subjected to different hormonal levels.

Generally, the research shows that close manipulation of auxin-to-cytokinin ratios can greatly boost root elongation in tomato micropropagation, which is important in the next step, acclimatization and successful planting under an ex vitro environment.

Table 6, Annexure (F)

3.1.8. Root induction percentage

The root induction percentage of tomato pure lines rose significantly with the increase in the concentration of BAP and IBA. The comparison of ANOVA indicated the PGRs and pure lines were highly significant in their effect on the percentage of root induction, but not in their interaction. The positive induction of roots was noted in Pure line 1 (BL-1174) and Pure line 2 (Tinto) at T6 (3mg/L BAP + 2.5mg/L IBA), achieving 51.667% and 71.667 percent respectively. Conversely, the control treatments showed the lowest percentage of root induction of 16.5% in BL-1174 and 18.333% in Tinto (Figure 7).

The enhanced capacity to induce roots at increased PGR concentrations could be attributed to the synergistic effects of auxins and cytokinins; the former triggers adventitious root formation, and the latter balances shoot-root signalling to promote root formation. The observation is in line with the findings of Jehan and Hassanein (2013), who said that the higher the auxin-to-cytokinin ratio, the easier it is to form roots in tomato explants. Besides, the Tinto pure line had a higher percentage of root induction than BL-1174, meaning that genotypic variation in response to hormonal treatment.

These differences could be due to differences in the endogenous levels of auxin or the sensitivity of meristematic cells to the available PGRs. These results demonstrate the significance of maximization of hormone levels to achieve effective induction of roots, which is a vital process to achieve effective acclimatization and subsequent culture of in vitro regenerated tomato plantlets in an in vitro environment.

Table 7, Annexure (G)

3.1.9. Number of leaves per explant

Micropropagation of tomato pure lines BL-1174 and Tinto showed that the concentration of BAP and IBA played a significant role in increasing the number of leaves per explant at high concentrations of BAP and IBA. The results of analyses of variance demonstrated that both PGRs and pure lines had a very significant effect on the leaf production, but not their interaction. T6 (3 mg/L BAP + 2.5 mg/L IBA) had the highest number of leaves of 5.5 in BL-1174 and 8 in Tinto. The lowest number of leaves, on the other hand, was in the control treatments, 2.3333 leaves in BL-1174 and 2.5 leaves in Tinto (Figure 8).

The higher number of leaves with the higher level of PGRs could be explained by the synergistic effect of cytokinins and auxins when the concentration of BAP stimulates the activity of cell division and meristem in the shoots, and the concentration of IBA ensures adequate differentiation and growth of leaves.

This physiological reaction is in agreement with Yang et al. (2001), who indicated an increase in the leaf proliferation in tomato explants with optimal cytokinin auxin ratios. Moreover, the Tinto pure line generated more leaves than BL-1174, which indicated a genotype-specific reaction to hormonal treatments, presumably because of the variation in the endogenous cytokinin levels and leaf primordia sensitivity. These findings have highlighted the necessity of establishing the best PGR concentrations to facilitate vegetative growth, which is crucial in obtaining the production of strong plantlets that can be taken through acclimatization and later ex vitro establishment.

Table 8, Annexure (H)

3.1.10. Internode distance

Micropropagation of tomato pure line BL-1174 and Tinto showed that the internode distance was correlated with the concentration of BAP and IBA. The analysis of variance revealed that the effect of both PGRs and pure lines on the internode elongation was extremely significant despite the absence of a statistically significant value of the interaction between these factors. BL-1174 at T5 and T6 (1.4333 cm) and Tinto at T6 (1.3833 cm) had the longest internodes, and the control treatments had the shortest internodes, with 0.9167 cm at T5 and T6, and 0.8333 cm at T6, respectively (Figure 9). The effect of higher PGR levels in increasing the length of internodes can be explained by the stimulatory action of cytokinins on cell division, stem cell elongation, and auxins such as IBA on cell division and vascular differentiation, which makes the nodes spaced further apart.

This finding is congruent with the findings by Yang et al. (2001), who suggested that similar trends in tomato explants with high cytokinin-auxin ratios did exist. Also, the BL-1174 pure line had slightly longer internodes as compared to Tinto, and this may imply a genotype-based reaction to hormonal therapies, which might be due to inborn variations in growth hormone reactivity and stem elongation ability. The results of these studies are significant to streamline the plant architecture during the in vitro propagation since the proper internode length may affect the further shoot and leaf growth, and plant vigour, all of which are imperative in the success of the subsequent acclimatization.

Table 9, Annexure (I)

3.1.11. Number of roots per explant

Micropropagation of tomato pure lines BL-1174 and Tinto revealed that the roots per explant increased with the increase in BAP and IBA concentrations. The analysis of variance indicated the highly significant effect of both PGRs and pure lines on the number of roots, but the interaction between the two factors was not statistically significant. In the case of BL-1174, T6 had the highest number of roots per explant (2.833) and Tinto (3.833), whereas the lowest number was at the control treatments with 1.1667 roots per explant in both cases (Figure 10).

It is possible to explain the progressive rise in the number of roots with the rise in PGR concentrations as having been caused by the promotional influences of the auxin-like plant growth regulators, including IBA, in root initiation and elongation and

the levelling influence of the cytokinin-like plant growth regulators, including BAP. This agrees with Singh et al. (2010), who stated that there was better root development in tomato explants in favourable ratios of auxin-cytokinin.

Though our findings are not consistent with those of Jamous and Abuqaoud (2015), who found otherwise trends in root induction under equivalent conditions, the response observed as a result of the genotype suggests that BL-1174 and Tinto are not sensitive to PGRs in the same way, and this is probably because these two-root meristem are inherently different in terms of their activity. These results demonstrate the relevance of the optimization of the level of auxin and cytokinin to enhance root development during in vitro propagation, which is essential to obtain successful acclimatization and further growth under ex vitro conditions.

Table 10, Annexure (J)

3.1.12. Survival percentage

The results of micropropagation of tomato pure lines BL-1174 and Tinto revealed that a higher concentration of BAP and IBA led to an increase in the percentage of germination. Analysis of variance showed that the effect of pure lines on the survival percentage was not significant, and the relationship between PGRs and the interaction between pure lines and PGRs was highly significant. The percentages of survival were the highest in T6 (3mg/L BAP + 2.5mg/L IBA), and it was 61.56768 in BL-1174 and 68.333 in Tinto, and the lowest percentages were in control treatments, 18.33 in BL-1174 and 15 in Tinto (Figure 11).

This is due to the increase in survival under the high concentrations of PGR, which is possible due to the synergistic action of the cytokinins and auxins, which leads to an increase in shoot and root development. BAP enhances the proliferation and chloroplast differentiation of the shoot, whereas IBA facilitates the formation of roots, which are required to guarantee enhanced plantlet wellbeing and acclimatization to ex vitro conditions. Genotype-specific tolerance and responsiveness to in vitro conditions were demonstrated by the Tinto pure line with increased survival over BL-1174, which could be attributed to the variation in endogenous hormone concentrations and meristematic activity. Though the acclimatization and field establishment were not directly measured in this experiment, the in vitro regenerated plantlets had well-formed roots, healthy shoots, and high percentages of survival, especially in Tinto, which are important markers of successful ex vitro transfer. Other reports on tomato have indicated that acclimatization of plantlets with regenerated plantlets in similar in vitro conditions succeeded at 7085% when transferred to sterile soil or peat-based mediums, keeping humidity and temperature constant (Singh et al., 2010; Raza et al., 2020).

This implies that optimal BAP and IBA levels not only contribute to an increase in vitro survival, but they also have high potential for greenhouse hardening and the following establishment in the field. Future studies ought to be done on ex vitro acclimatization, transfer to greenhouse, and field performance to determine the stability of growth, yield potential, and agronomic uniformity of regenerated plants.

Table 11, Annexure (K)

3.1.13. Plant height

In micropropagation of tomato pure lines BL-1174 and Tinto, it was noted that the height of the plants rose with an increase in the level of the BAP and IBA.

Evaluation of variance revealed a very high level of significance of both PGRs and pure lines in the plant height. T6 appeared to have the highest plant height of 3mg/L BAP + 2.5mg/L IBA, with BL-1174 and Tinto recording 2.6167cm and 2.65cm, respectively (Figure 12). Increased height of the plant with an increase in PGR level can be explained by the synergistic effect of cytokinins and auxins, whereby the increase in height of the plant and meristem activity caused by the BAP, and an increase in root growth caused by IBA, where the root achieves structural support to enable the shoot growth. Tinto pure line was marginally taller than BL-1174, which indicates that genotype response to hormonal interventions is specific, which could be attributed to variations in the level of endogenous hormones and the rate of growth.

In the study by Raza et al. (2020), the height of the plant was higher, and this could be attributed to the fact that the measurement was done at six leaves stage of plant growth, and the current study measured the plant height at 23 leaf stage, which was an early growth stage of the plant. The findings indicate that the optimal levels of BAP and IBA can enhance not only the shoot growth but also the general well-being of the plantlets, thus enabling successful acclimatization and the eventual growth of the plantlets in vitro.

Table 12, Annexure (L)

Experiment No 2

In the study, the callus induction was used in tomatoes to induce mutations in the tomato species to produce new varieties of tomatoes resistant to cold, drought and salinity.

3.2 Callus Induction of Tomato

Callus induction was performed in tomato pure lines BL-1174 and Tinto to facilitate in vitro mutagenesis, which can be utilized to develop new tomato varieties with enhanced tolerance to abiotic stresses such as cold, drought, and salinity.

3.2.1 Days to Callus Induction

The induction of callus in tomato pure lines BL-1174 and Tinto was made successful. The value of analysis of variance showed that the number of days to induce callus was highly affected by PGRs, their genotypes, and the interaction of the two. Mean comparison between PGRs and genotypes (Figure 13) showed that the days to callus induction were reduced significantly by increasing the concentration levels of the plant growth regulators.

The highest number of days was found in T1 (low PGR concentration) at 45 days in both cases of inducing callus in BL-1174 and Tinto, and the lowest number of days was found in T6 (high PGR concentration) at 37 days in both cases. This decrease in the number of days to callus induction under high PGR concentration may be due to the synergistic effect of the auxins and cytokinins, in which the 2, 4-D facilitates cell dedifferentiation and callus formation, whereas BAP facilitates cell proliferation and shoot meristem action.

Also, the genotype-specific responses that were evident indicate a slightly higher response of Tinto to PGR supplementation compared to BL-1174, which could have been a result of natural variations in endogenous hormone concentrations and tissue regenerative ability.

These results indicate that the optimization of PGR levels is a key to effective callus induction, which is a background procedure to further uses of tissue culture, such as mutation breeding and genetic transformation.

Table 13, Annexure (M)

3.2.2 Callus induction percentage

Tomato pure lines BL-1174 and Tinto induction of callus was successfully attained. The results of the analysis of variance showed that the effect of PGRs and genotypes on the percentage of callus induction was very significant, but the interaction was not significant. Comparison of means of PGRs and genotypes (Figure 14) revealed that the percentage of callus induction rose significantly with the increase in the concentrations of plant growth regulators in both genotypes. The highest percentages of callus induction were recorded at T6, with the highest percentages being 76.66% in BL-1174 and 90% in Tinto, and the lowest percentages of 10 and 13, respectively. This increased callus induction in high levels of PGR could be attributed to the role played by 2,4-D and BAP synergy, in which the former induces dedifferentiation and proliferation of the cells, and the latter augments mitotic activities and facilitates the division of the meristematic cells, thereby resulting in a healthy callus.

Genotype-specific variations were also noted, whereby Tinto showed a higher percentage of callus induction when compared to BL-1174, which is probably a result of natural variation in the levels of endogenous hormones, tissue plasticity, and regeneration potential. These results prove that to ensure an efficient induction of callus, it is important to optimize the levels of PGRs, which are important in the downstream use in mutation breeding, genetic transformation, and making tomatoes more stress-tolerant. Our findings are comparable to other reports in which genotypes of tomatoes had an inconsistent reaction to the induction of callus and regeneration (Baste et al., 2007; Chaudhry et al., 2007).

Table 14, Annexure (N)

3.2.3. Callus area

Induction of callus in tomato pure lines BL-1174 and Tinto was also successful, and analysis of variance showed a significant effect of PGRs on the area of the callus. The effects of genotype and the combined effect of PGRs and genotype were not statistically significant, whereas increasing concentrations of IBA and BAP in both genotypes were associated with an expansion in the area of callus. Mean comparison between PGRs and genotypes (Figure 15) revealed that there was a progressive increment of the callus area as the concentration of both pure lines of plant growth regulators was increased.

The largest callus area was detected in T6 at 1.66 cm 2 in the case of BL-1174 and 1.75 cm 2 in the case of Tinto, respectively, and the smallest callus area was determined in T1 at 0.5167 cm 2 and 0.6333 cm 2 in the case of BL-1174 and Tinto, respectively. The growth of callus under high levels of PGR is explained by the joint effect of auxins and cytokinins, so that IBA induces cell elongation and division, and BAP induces mitosis, resulting in the increased proliferation of tissues. Genotype-specific differences were also noted where Tinto generated a somewhat bigger area of callus than BL-1174, which could be attributed to differences in the levels of endogenous hormones and intrinsic tissue regenerative ability.

These data demonstrate the need to maximize the PGR concentrations to obtain the optimal callus growth, which is necessary to apply it to downstream applications, i.e., somaclonal variation, genetic transformation, and the creation of stress-tolerant tomato lines. We have found that our results are in line with those of other studies that have shown genotype-

dependent callus area responses in tomato (Baster et al., 2007; Osman et al., 2010).

Table 15, Annexure (O)

3.2.4 Genetic Stability of Regenerated Plantlets

The present study did not involve any molecular or cytological analysis; however, based on the pathway of regeneration used, it is plausible to consider the regenerated plantlets as genetically stable. Micropropagation in the present protocol was mainly accomplished by nodal explants and direct organogenesis as opposed to extended callus-mediated regeneration, which is known to reduce somaclonal variation. Clonal faithfulness and genetic uniformity Clonal fidelity and genetic uniformity Clonal f Genetic stability of micro propagated tomato plants obtained using nodal explants has been confirmed by several studies using molecular (RAPD, ISSR and SSR) and cytological (counting of the chromosomes and flow cytometry) markers (Bairu et al., 2011).

The regenerated plantlets in the current experiment had normal morphological characteristics, a similar growth pattern and constant growth characteristics, and these further confirm the possibility of genetic stability. Nonetheless, as a future study and possible commercial usage, it can be suggested that molecular marker-based validation and cytological verification of clonal fidelity should be conducted. This validation will increase the confidence of uniformity and performance of regenerated plants when subjected to ex vitro and field conditions (Sharma et al., 2020).

4. Conclusion

This paper addresses the optimization of callus induction and micropropagation regimes of pure-line tomato (Solanum lycopersicum L.), which is a crop of agricultural and nutritional importance worldwide. In Pakistan, tomatoes are a significant source of nutrition and income, as well as in the global vegetable market, which is based on tomatoes as a major food source. Even though tomatoes have commercial and nutritional potential, some challenges like the absence of disease-resistant and high-yielding varieties, environmental stresses, pests and disease pressures are affecting tomato cultivation. These constraints have not been adequately addressed using conventional breeding techniques, and this has forced the incorporation of biotechnological tools that include plant tissue culture. The use of plant tissue culture offers an effective and efficient way of obtaining homogenous, disease-free and genetically identical plants, which is critical in modern agricultural practice. Although advanced biotechnological methods like the induction of somatic embryos, genetic transformation, and mutation breeding utilize callus induction, micropropagation is an effective method of increasing elite cultivars to produce commercial tomatoes. This experiment has produced a successful protocol of in vitro regeneration of tomatoes in pure-line tomatoes, which can make a great contribution to both future research and commercial tomato growing. Two experiments were carried out in the Plant Tissue Culture Cell, Department of Horticultural Sciences, UAF, namely: callus induction and micropropagation. Some of the parameters measured were the number of leaves, internodal distance, height of the plant, survival percent, the number of roots per explant, the number of shoots per explant, days to callus induction and area of the callus. Diverse ratios and levels of plant growth regulators (PGRs) BAP, NAA, IAA, and IBA were tested in

Murashige and Skoog (MS) medium to determine the effects of various explants, including root segments, shoot apices, and nodal segments. It was observed that the auxin and cytokinin hormonal ratio played a significant role in the regulation of organogenesis and morphogenesis. A higher cytokinin/auxin ratio was associated with the induction of the shoot, higher levels of auxin stimulated callus growth and root development, which emphasized the necessity of fine-tuning the hormone balance in the tissue culture. The findings assured that the nature of explant and the level of PGRs also had a significant effect on the amount and quality of callus induction and plant regeneration. The most effective combinations of BAP and NAA caused the best callus formation, whereas the combination of BAP and kinetin or IAA caused the best efficiency of shoot regeneration. The protocol that was developed allowed rapidly multiplying pure-line cultivars, generating plantlets of normal morphology and presumably genetic stability, implying that this tissue culture system will be reliable in preserving the genetic integrity of the material that is being propagated. These streamlined tissue culture guidelines have great practical consequences. They offer a consistent means of generating high volumes of pathogen-free planting material, which sustains high yield in the case of commercial agriculture. Such protocols in scientific research provide a platform to research genetic transformation, molecular breeding programs, and develop tomato cultivars that are more resistant to stress and disease. Moreover, effective callus induction and regeneration of plants enable the study of somaclonal diversity and the initiation of desirable characteristics using gene editing or transgenic systems, which leaves possibilities of enhanced crop development. The paper shows that the efficient optimization of PGRs, choosiness and culture milieu can greatly augment the efficacy of in vitro regeneration in pure-line tomatoes, bridging the research and the practice divide in the domain of agriculture and biotechnology.

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Table 1, Annexure (A)

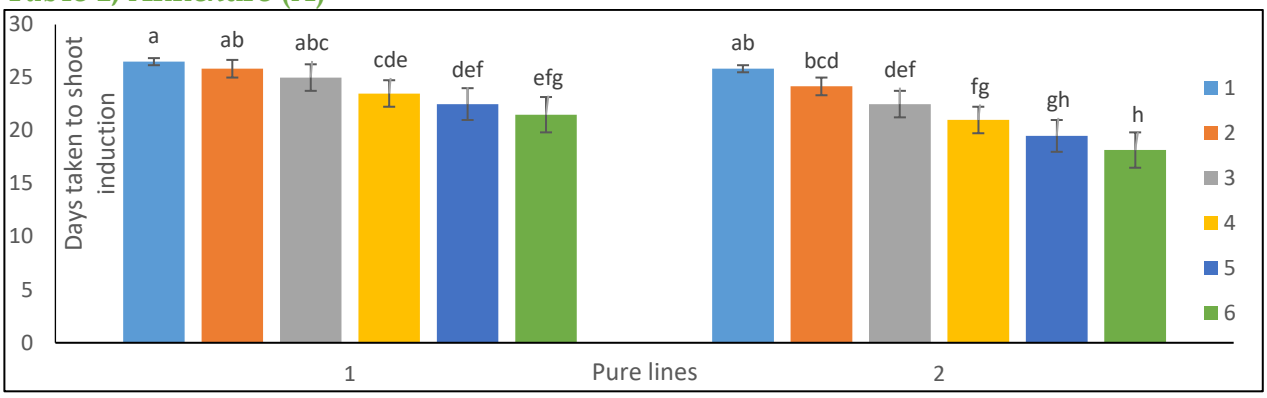

Figure1: Number of days taken to induce shoot induction in pure line 1 (BL-1174) and 2 (Tinto). T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 2, Annexure (B)

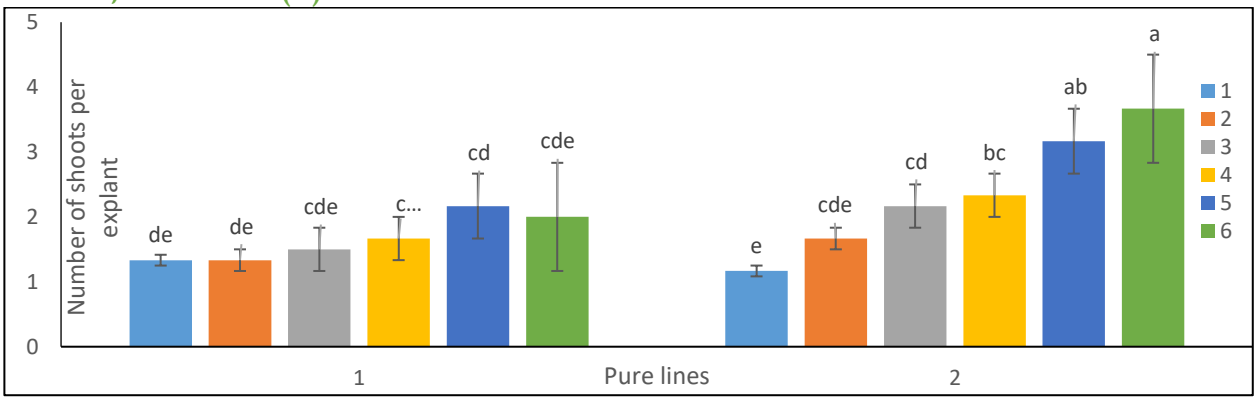

Figure 2: Number of shoots per explant in 1 (BL-1174) and 2 (Tinto) pure lines. T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 3, Annexure (C)

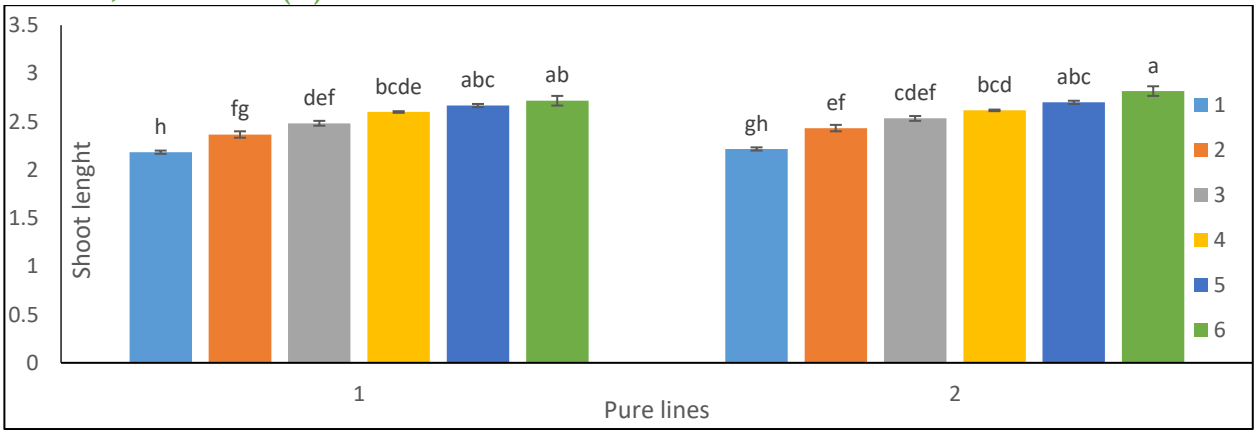

Figure 3: Shoot length in pure line 1 (BL-1174) and 2 (Tinto). T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Mean followed by a different superscript
letters differ significantly (P ≤ 0.05).

Table 4, Annexure (D)

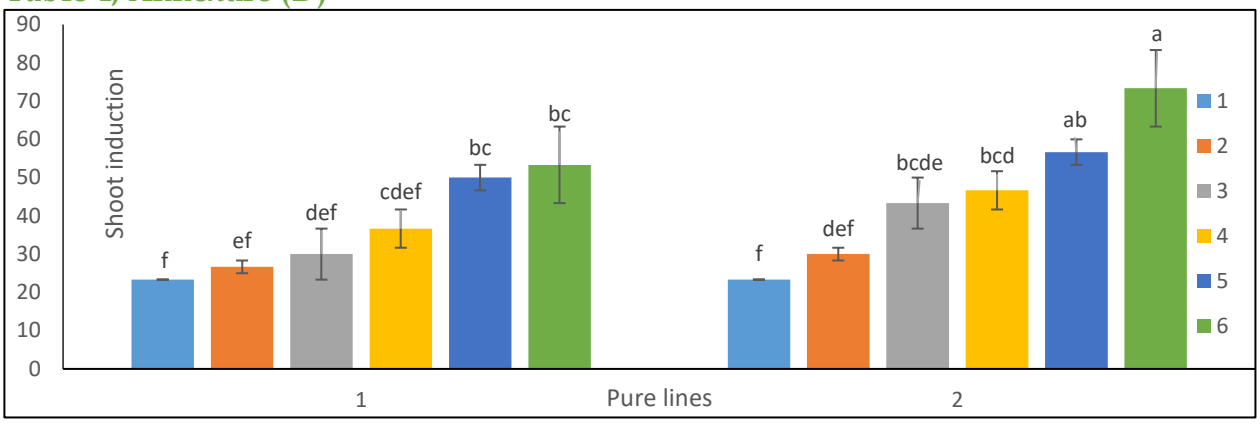

Figure 4: Shoot induction percentage in 1 (BL-1174) and 2 (Tinto) purelines. T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 5, Annexure (E)

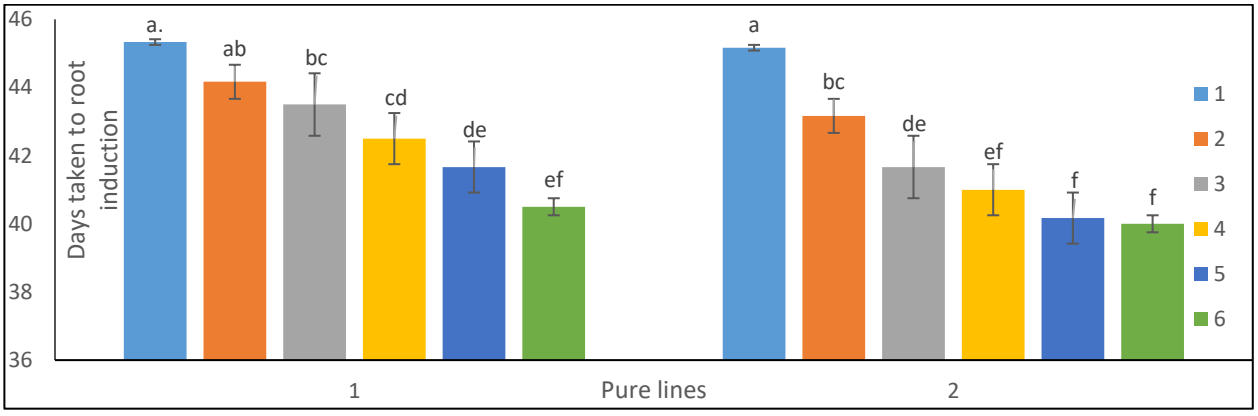

Figure 5: Days taken to root induction in 1 (BL-1174) and 2 (Tinto) pure lines. T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 6, Annexure (F)

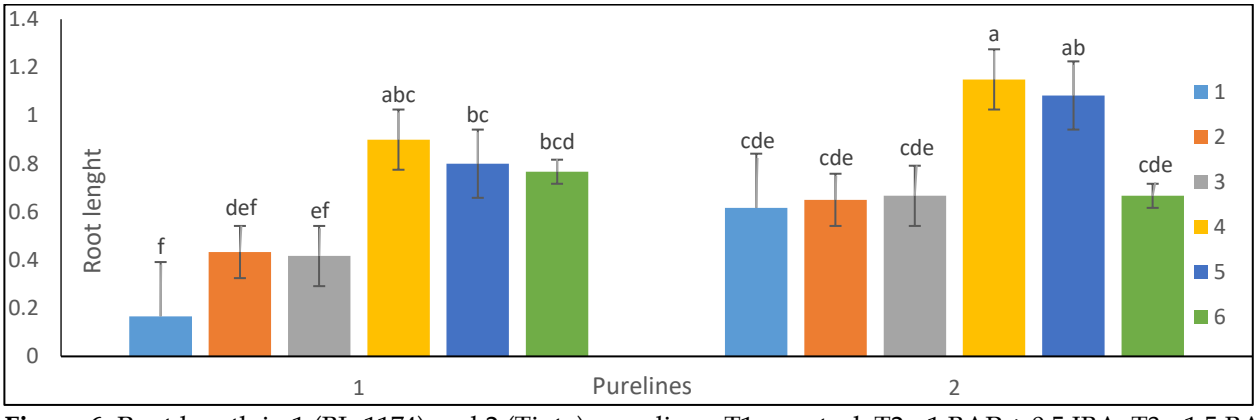

Figure 6: Root length in 1 (BL-1174) and 2 (Tinto) pure lines. T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 7, Annexure(G)

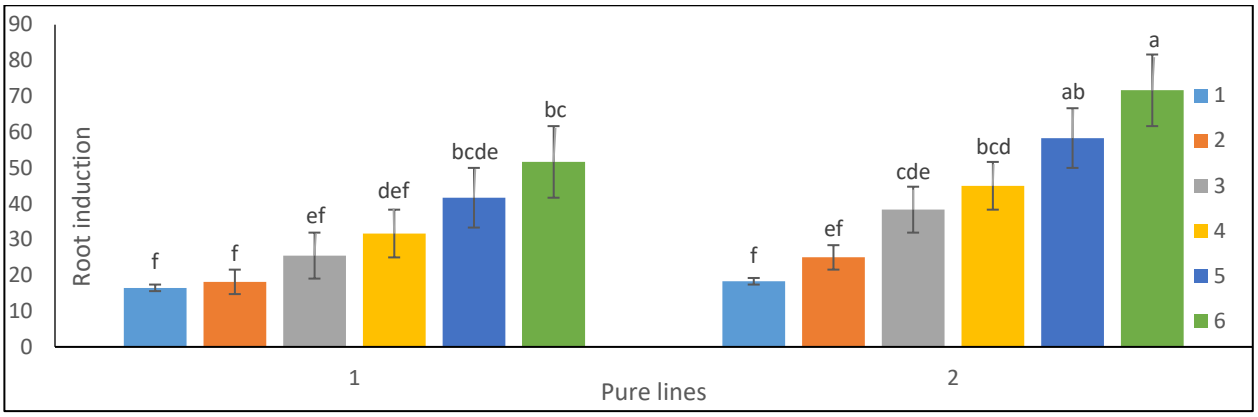

Figure 7: Root induction percentage in Pure line 1 (BL-1174) and 2 (Tinto). T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 8, Annexure (H)

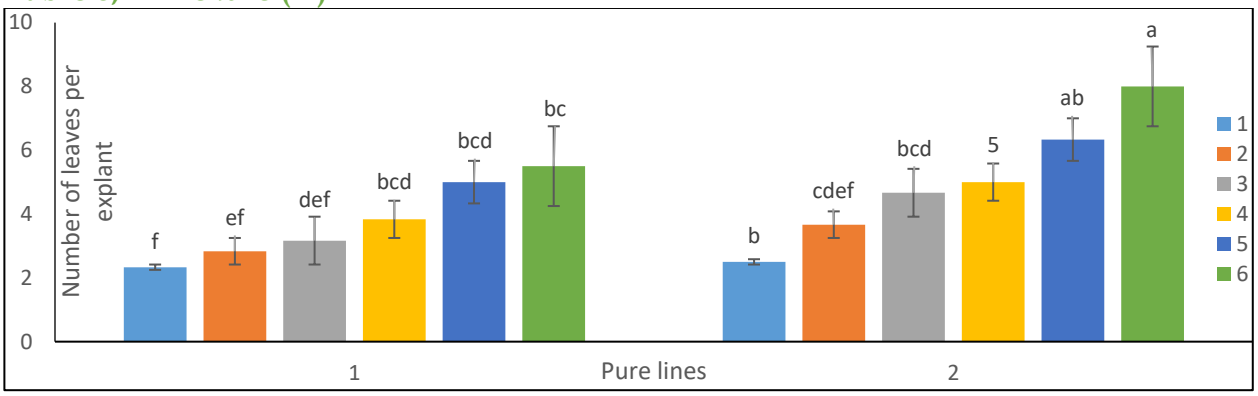

Figure 8: Number of leaves per explant in Pure line 1 (BL-1174) and 2 (Tinto). T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 9, Annexure (I)

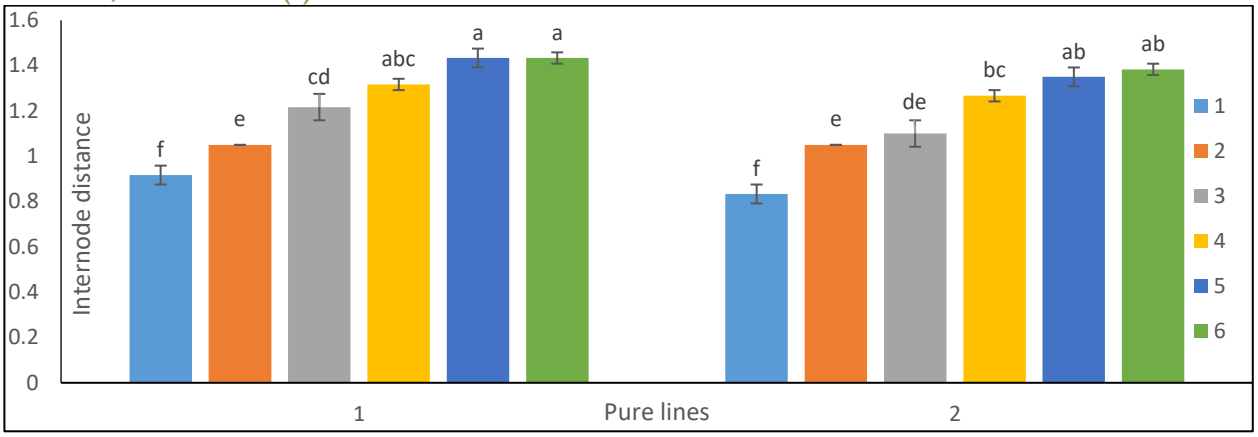

Figure 9: Internodal Distance in Pure line 1 (BL-1174) and 2 (Tinto). T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 10, Annexure (J)

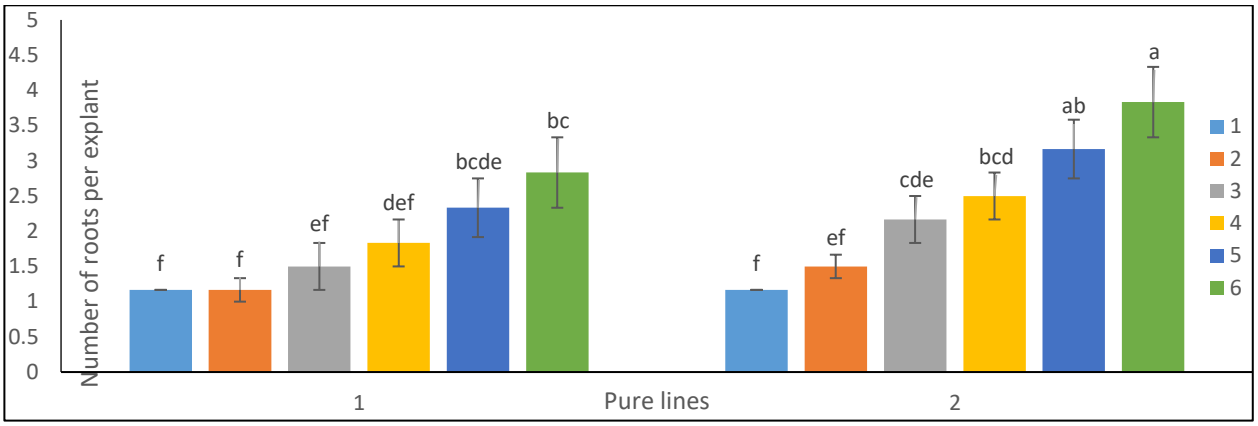

Figure 10: Number of roots per explant in Pure line 1 (BL-1174) and 2 (Tinto). T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 11, Annexure (K)

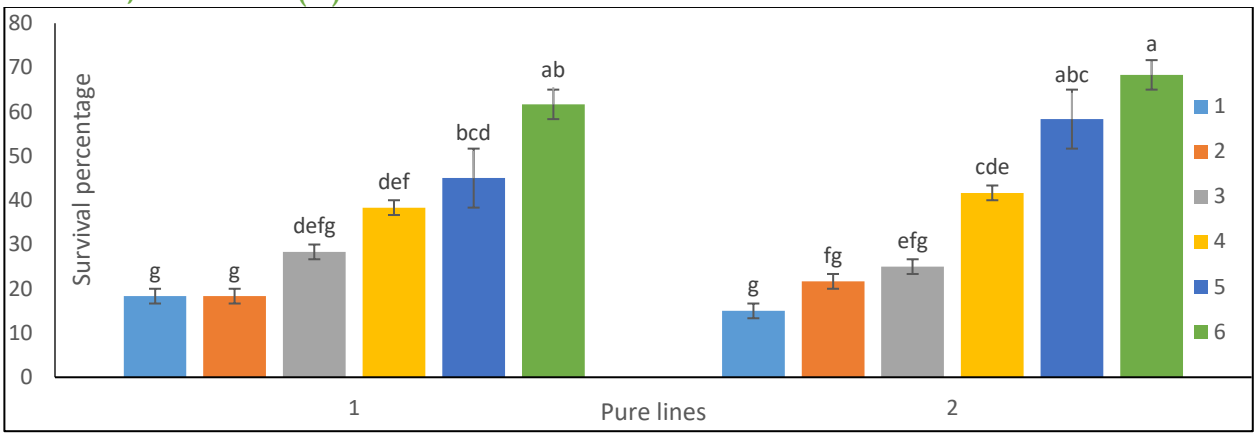

Figure 11: Survival Percentage in Pure line 1 (BL-1174) and 2 (Tinto). T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 12, Annexure (L)

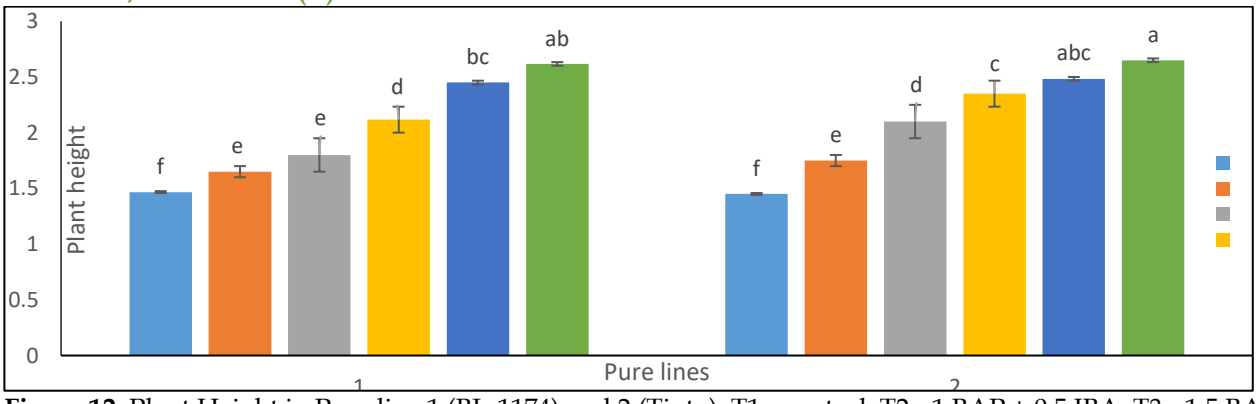

Figure 12: Plant Height in Pure line 1 (BL-1174) and 2 (Tinto). T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 13, Annexure (M)

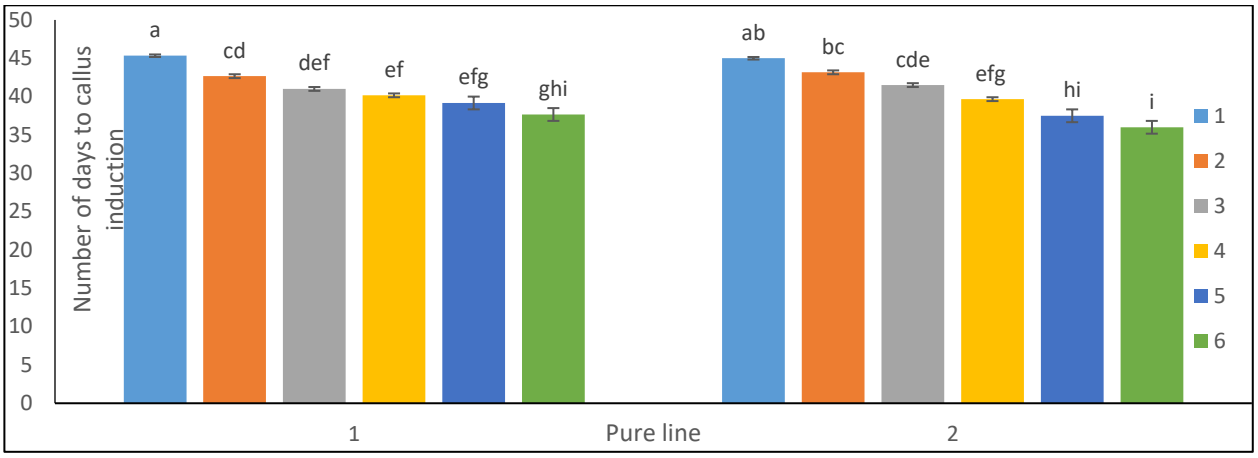

Figure 13: Number of days to callus induction in Pure line 1 (BL-1174) and 2 (Tinto). T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 14, Annexure (N)

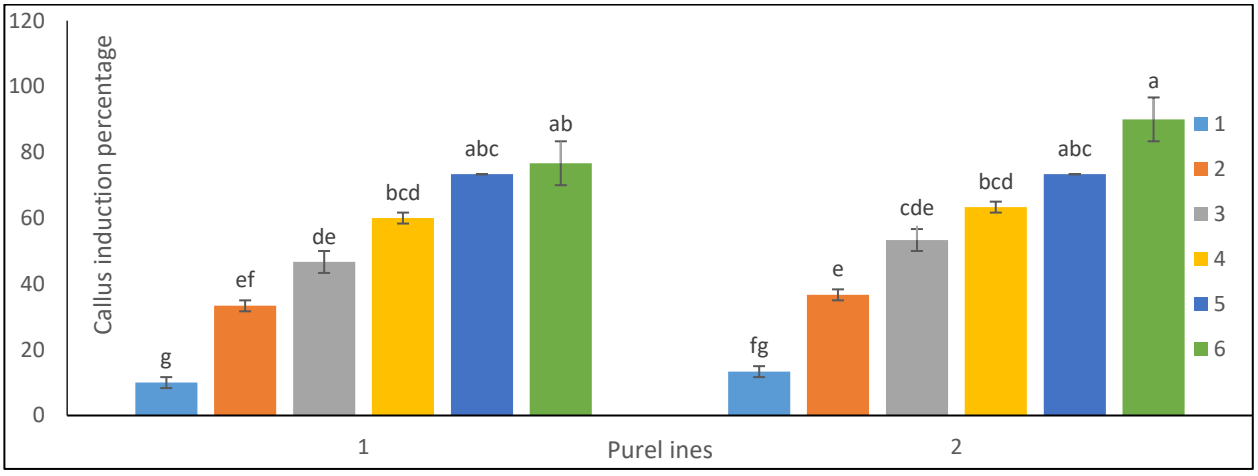

Figure 14: Callus induction percentage in Pure line 1 (BL-1174) and 2 (Tinto). T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).

Table 15, Annexure (O)

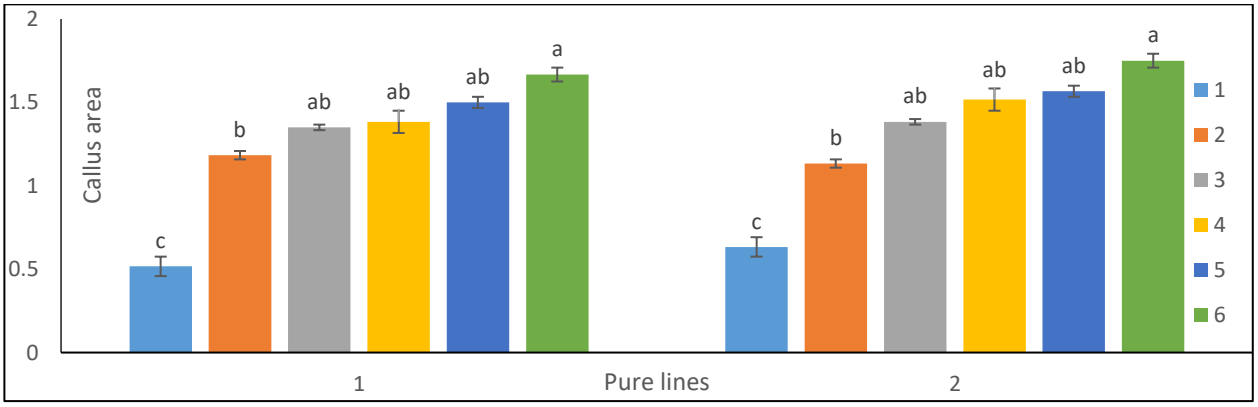

Figure 15: Callus area in Pure line 1 (BL-1174) and 2 (Tinto). T1= control, T2= 1 BAP + 0.5 IBA, T3= 1.5 BAP + 1 IBA, T4= 2 BAP+ 1.5 IBA, T5= 2.5 BAP+ 2 IBA, T6= 3 BAP+ 2.5 IBA. Means followed by different superscript letters differ significantly (P ≤ 0.05).