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RESEARCHARTICLE

Trends in Biotechnology and Plant Sciences

In vitro Callogenesis and Regeneration of Tomato (*Lycopersicon esculentum* Mill.)

Ghausia Aziz[1], Hina Ghaffar[1], Syeda Anam Akram[1], Imran Shehzad[1], Hafiz Muhammad Abdullah[1], Amna Mughal[1], Muhammad Nisar Anjum[1] and Maria Rehman[2*]

[1]Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture Faisalabad.
[2]Department of Biotechnology, University of Sargodha

Correspondence
mariarehman577@gmial.com

Abstract
Tomato (*Solanum lycopersicum* L.), is ranked at second in vegetables after potato. Although its demand and production is growing progressively, yet; biotic & abiotic stresses like high salt concentration, water shortage, high temperature, infectious diseases and nutrient deficiency are the main causes of its yield reduction. Many tissue culturing protocols and techniques have been practiced to develop such protocols which can enhance its rate of revival and prolong time of regeneration. Two cultivars of tomato Naqeeb and Nadir were selected to get explants and MS medium containing plant growth regulators including. Zeatin and IAA showed enhancement in regeneration frquency. Statistics of 192 explants was calculated after 4 weeks of culturing. Research was carried out on the basis of Trifactorial setup to study the result of genotype, different explant resources and media prepared and their collaborations individually & in combination, on *in vitro* competency of regeneration (count of shoots per ex-plant). Outcomes were calculated by means of Analysis of Varience, by comparing both varieties. Overall superiority of Tomato variety Naqeeb was observed. Fischer's LSD test (Least Significant Difference) was used to calculate dissimlarities amongst the central values that are assumed arithmetically significant.

KEYWORDS
in-vitro Regeneration, Tissue culture, Plant growth regulators, Explants, Culture condition

1 | INTRODUCTION
The tomato (*Lycopersicon esculentum* Mill.), a member of the Solanaceae family with chromosome number 2n = 2x = 24, a herbaceous plant originated to western South America, is one of the most widely grown vegetable species in the world. Although the tomato was thought to have originated in a specific region of South America, Mexico is most likely where it was originally domesticated (Salim et al. 2020). Native to western South America, wild tomato plants can still be found in the Galapagos Islands, central Ecuador, northern Chile, Peru, and along the coast and high Andes. Based on the most recent statistics available, more than 5 million hectares were set aside for tomato production in 2019. More than a million hectares were grown in China, followed by India, Turkey, the United States, and Egypt. Together, these countries account for more than 60% of global tomato output (Caruso et al. 2022).

The number of tomatoes produced worldwide was 186,821,216. They are eaten raw in salads or cooked in soups, sauces, and other meals. They can also be processed to make ketchup, purées, pastes, and juices. Dried and canned tomatoes are processed foods with significant economic value. They are rich in minerals, essential amino acids, carbohydrates, and dietary fibers, and they also include a variety of substances that are good for you, such as vitamins, carotenoids, and phenolic compounds (Tchonkouang et al. 2022). Tomato fruit consumption is regarded as a healthful diet that may lower the risk of osteoporosis, cardiovascular disease, and cancer (Campestrini et al. 2019). Regular tomato eaters were shown to have lower blood pressure, cholesterol, blood sugar, heart disease, and damage to their cells. The abundant

source of lycopene is tomato fruit. One kind of carotenoid found in tomatoes, processed tomato products, and other fruits is lycopene (Kumar et al. 2020). Tomatoes contain a variety of compounds that are employed in health products, such as flavonoids, carotenoids, glycosides, steroidal alkaloids, and fatty acid derivatives (Wang et al. 2023). Tomato seed oil and essential oil are particularly useful in skin care purposes (Waheed et al. 2020). Globally, biotic and abiotic stresses have an impact on agricultural productivity, which lowers crop yields in many cases. The two most dangerous abiotic stressors are heat and drought, particularly in nations with warm climates. Insects, bacteria, fungus, viruses, and other plant pathogens are typically stimulated by heat and drought adversely effect the tomato yield (Mishra et al. 2022; Chattha et al., 2024; Zafar et al., 2024). Tomato bushy stunt virus (TBSV) and tomato mosaic virus (ToMV) are two of the primary agents of such illnesses that reduce tomato crop output globally. Tomato is also considered one of the ideal crops for *in vitro* investigation because of its small chromosome number (2n=24) and a huge data about the genetics of tomato genetics. To control certain biotic and a biotic stress, a competent and rapid *in vitro* tissue culture scheme is very significant for constant enhancement in tomato through transformation of agricultural point of view vital DNA sequence. Numerous biotechnological applications arise from the adjustment of culture conditions for callus development and tissue regeneration of various tomato genotypes. In this study, we examined the impact of varying zeatin and indole-3-acetic acid concentrations on tomato cultivars' cotyledon explant regeneration (Yaroshko et al. 2023).

Tomato has been used widely as a model crop for their genetic upgrading. Because of the commercial worth of tomato, *in vitro* regeneration of cultured tomato *(Lycopersicon esculentum* Mill.) has got a great importance in research and its liability for better enhancement through genetic planning. In tomato, the tissue culture job is really tough because particular hormones are required for each variety made and scientists have to treat all genetic varieties independently (Sandhya et al. 2022). Several laboratories are conducting certain experiments on tomato to employ the food quality via studying transformation methodologies. But transformation methodologies for tomato can depending upon the genotype of tomato to be used (Wan et al. 2018). Different explants, including the cotyledon, hypocotyl, and leaf of two tomato genotypes, were used to enhance the transformation process. The current work may help create better tomato genotypes by transferring desired features and using precise genome editing techniques (Sandhya et al. 2022).Numerous constant elements include tomato type, culture, climate, harvest ripeness, and long-term storage conditions (Raheem et al. 2019). Using genome editing technologies, it is essential to create an effective genetic transformation and plant regeneration system in various tomato genotypes that is resistant to biotic and abiotic challenges and produces high yields (Van Eck, 2020). Though many trials have been carried out to develop *in vitro* regeneration protocol for numerous business-related tomato genotypes consuming a huge array of ex-plants, such as leaf discs, stem and leaf, hypocotyl and cotyledon and protoplasts on different growth medium. Out of these, cotyledon ex-plants was documented to be the greatest reactive ex-plant for *in vitro* regeneration and transformation in different tomato genotypes. MS basal media is proved to be the widely used media for tomato regeneration. The main aim of this work was the relative analysis of the effect of varying growth media composition on the basis of different levels of hormones on two tomato cultivars Naqeeb and Nadir on in vitro regeneration, using different explant.

2 METERIALS AND METHODS

Plant Material

Seeds of Tomato cultivars "Naqeeb" and "Nadir" were attained from Vegetable Research Institute (VRI), Ayub Agricultural Research Institute (AAR1), Faisalabad.

Seed Sterilization

Seeds were soaked in 70% ethanol for 1-2 minutes and then in 50% bleach along with few drops of tween-20 for five minutes. In the last, 4-5 times washing was carried out with sterile distilled water. After washing, seeds were grown in autoclaved 180 ml tissue culture glass bottles having solidified MS0 media, placed them in growth room at a temperature of 25°C ± 2°C providing 16/8 hrs light: dark via cool white fluorescent lights.

Evaluation of Medium and Culture Setting

MS basal medium (Murashige and Skoog, 1962) was used for callus initiation and subsequent *in vitro* regeneration. Autoclaved distilled water was used to prepare solutions of vitamins and Plant Growth Regulators (PGRs). Sucrose used as a source of carbon at the rate of 30.0 g/L. The pH adjusted at 5.8 with addition of an alkali NaOH or acid HCl before the addition of Gelling powder at the rate of 2.66 g/L as a solidifying agent. All the glass jars containing media and seeds were placed in the Growth room where conditions were optimized at a temperature of 25°C ± 2^{o}C in 16/8 hrs Light: dark conditions, at light intensity of 90-100 micro mol/m^{2}/ s. These media combinations

along with mentioned levels of PGRs also include sucrose at the rate of 30g/L; MS salts at the rate of 4.33g/L, MS vitamins at the rate of 1ml/L, myo-inositol at the rate of 100 mg/Land Gellen gum powder at the rate of 2.66 g/L.

Selection of Explants

Various explants including cotyledons, hypocotyls, leaves and roots from aseptically germinated seeds were used as explants were cut into small pieces and placed on MS media fortified with combinations of growth regulators.

Callus/Direct Shoot Initiation

Tomato plants germinated in aseptic *in vitro* environment, of 10-12 days old were used for ex-plant collection. From these plantlets, 6 days and 8 days old cotyledons, hypocotyls, roots and leaves were excised and were cultured into petri plates containing above mentioned combination of PGR's for *in vitro* callus introduction, direct shoot initiation and regeneration.

Shoot Induction by using Different Concentrations

Calli from various explants including intact cotyledons, hypocotyls, leaves and roots excised from, seedlings germinated in sterile environment were used for *in vitro* shoot induction and regeneration. Each glass jar having 100ml MS medium supplemented with different concentrations of Zeatin / IAA for shooting were to be placed in growth room (Table 1.2).

Experimental Design and Data Analysis

Callus initiation & plant restoration response of both varieties "Naqeeb" and "Nadir" was performed by applying Analysis of Variance (ANOVA). The Standard errors of means (SEM) were planned and statistical impact between the mean values of both varieties was calculated by applying LSD test with 5% level of significance. The significance depends upon the P calculated where data shows the following rules for significance: Non-significant (P>0.05), Significant (P<0.05), Highly significant (P< 0.01).

3 RESULTS

A capable and reliable plant regeneration procedure is vital for the implementation of regular plant transformation methodologies. Utilization of totipotent cultivars, suitable ex-plants, proper media composition and culture conditions are of essential significance in maintaining a suitable plant tissue culture protocol.

Seed Surface Sterilization and Seed Germination for Ex-plant Collection

For sterilization, Seeds' surfaces were sterilized by consecutive soaking in 70% (vol. /vol.) ethanol for 1-2 minutes and 50% commercial bleach +1-2 drops of Tween-20 which serves as a wetting agent for 5 minute, then washed 4-5 times with autoclaved distilled water for 5 minutes. In these autoclaved glass jars, which include solid MS0 medium seeds were germinated (Fig. 1.1a). After 10-12 days of germination data was noted down. Highest germination (87.3%) with least infection (3.7%) and (9%) non-germinated for Naqeeb. (85%) germination, (6.3%) contamination and (8.2%) non-germinated for Nadir was obtained respectively as indicated in Table. 1.3. From these germinated seeds, leaves, cotyledons, roots and hypocotyls were excised to be used as ex-plants sources.

Table 1: Various culture media blend used for callus induction and regeneration

Sr. No.	Treatment Name	NAA : 6-BENZYL AMINOPURINE Conc (mg/L)	Sr. No.	Treatment Name	NAA : 6-BENZYL AMINOPURINE Conc (mg/L)
1	T1	0.5 : 1	6	T6	1:2
2	T2	0.5 : 1.5	7	T7	1.5: 1
3	T3	0.5: 2	8	T8	1.5 : 1.5
4	T4	1: 1	9	T9	1.5: 2
5	T5	1 : 1.5	0	T0	0 : 0

Table 2: Different combinations of Zeatin and IAA used to persuade the Shoot Regeneration

Sr. No.	Treatments	Media		
		MS (mg/L)	Zeatin (mg/L)	IAA (mg/L)
1	R1	MS	2	0.1
2	R2	MS	1.0	0.1
3	R3	MS	1.0	1.0

Table 3: Seed Germination of two varieties of tomato

Varieties	No. of seeds	Germinated	Non-germinated	Contamination
Naqeeb	110	96(87.3%)	10 (9%)	4 (3.7%)
Nadir	110	94 (85.5%)	9 (8.2%)	7 (6.3%)

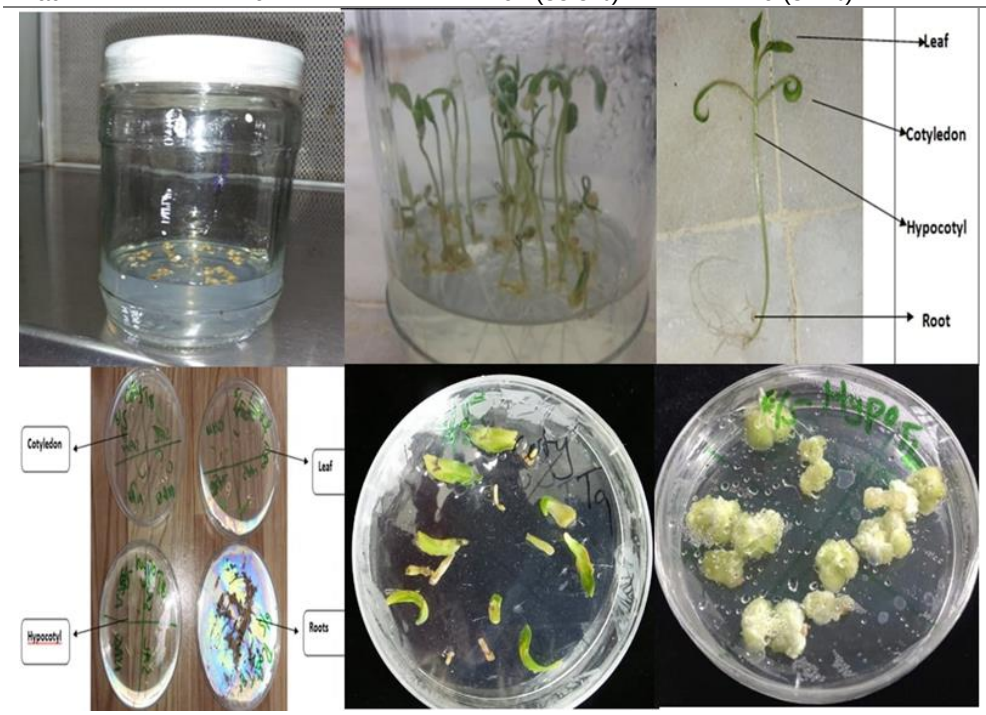

Fig. 1: In vitro regeneration stages A) *In vitro* tomato seed germination for ex-plant gathering B) Eight days old grown seeds of Tomato C) *In vitro* grown tomato plants showing different ex-plants D) Different explants cultured for callus induction E) Twelve- days old cultured for callus induction F) Three weeks old Callus from hypocotyls ex-plant.

In vitro Callus Induction and Regeneration

Inside the laminar flow, 10-12 days old *in vitro* grown seedlings, cotyledons, hypocotyls, leaves & roots were cut and cultured on regeneration medium (Fig. 1e). Cotyledons, leaves and primary roots cut with 3-4mm size were cultured onto MS basal medium augmented with 30g/L sucrose, 1ml/L mio-inositol, MS vitamin 1ml/L and Ms salts 4.33mg/L along with different concentration of 6-benzyl aminopurine and NAA (Table 1), PH was adjusted to 5.8 and 2.66 mg Phytagel was used to solidify the medium. After a period of 3 weeks of culturing, data was documented Fig. 1f). Treatment T9, while leaf disc was used as explant give maximum callus formation (77.8 %) and (72.2%) while cotyledon used as explant. While as (T1) treatment (0%) callus formation observed using root explant (Fig. 2).

All shoots were shifted independently to glass pots for root initiation. All jars containing 100ml MS basal media augmented with different concentrations of Zeatin /IAA (Table 2) for shooting were kept in growth room which was optimized for temperature at 25°C ± 2°C and light: dark conditions as16/8 hours respectively. All the plants were set aside on this medium in the growth room for two to four weeks till prominent shooting was to be observed.

Root Introduction from Regenerated Shoots of Tomato in *in vitro*

As regenerated shoots to be excised from explants were planned to split and be treated with 15mg/l IBA for two hours, afterward all shoots were to be shifted separately to autoclaved glass containers for root initiation. All glass pots contain 75 ml of 0.5MS media augmented with 0.2mg/l IBA and 0.25 mg/l 6-Benzyl Aminopurine for rooting. Plants were kept for 20-28 days (2-4 weeks) until strong rooting was to be observed. But unfortunately, all Calli from all explants of both varieties could not induce shoots, instead they all induced roots. Using cotyledon explants, a one-way ANOVA was conducted to evaluate the variations in root regeneration between the two tomato types, Nadir and Naqeeb. With an F-value of 242.50 and a P-value of 0.0001, demonstrate a very significant influence of genotype on root regeneration. The grand mean for root number per cotyledon explant was 28.4, with a coefficient of variation (CV) of 2.90%. According to the Least Significant Difference (LSD) test, genotype 1 (Naqeeb) produced significantly more roots (mean = 39.00) compared to genotype 2 (Nadir) (mean = 28.00). The difference in means demonstrates that Naqeeb consistently produced more roots than Nadir, with a significant T value of 2.776 at $\alpha = 0.05$. For hypocotyl explants, the ANOVA results also indicated a highly significant difference between the two varieties ($F = 138.00$, $P = 0.0001$), suggesting that genotype influences root regeneration from hypocotyl explants. The LSD test for hypocotyl explants showed a mean of 42.00 roots per explant for Naqeeb and 37.00 roots per

explant for Nadir. Both means fell into different groups (A for Naqeeb, B for Nadir), confirming a significant difference between the genotypes (SE = 0.8165, T-value = 2.776). The analysis of variance revealed that there was a substantial difference in root regeneration between the two tomato types, Nadir and Naqeeb,

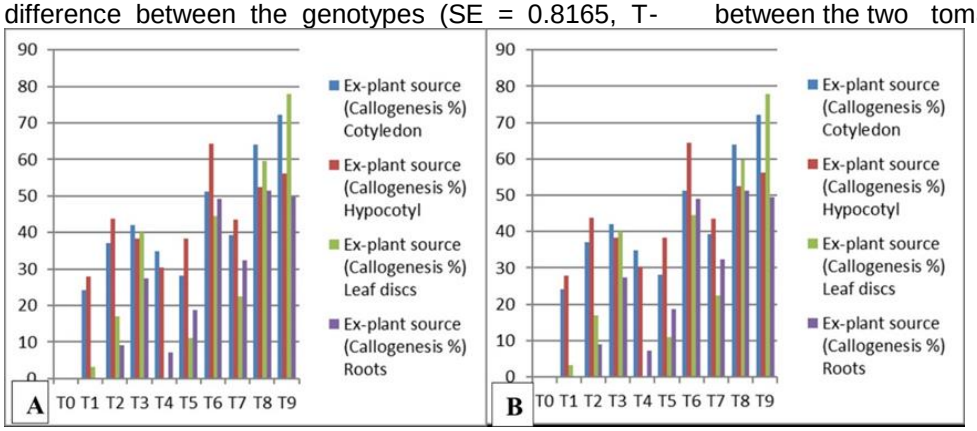

Fig. 2: (A) Harmone induced Callogenesis from explants of tomato variety Naqeeb (T0 control group, rest have different treatments). (B) Callogenesis from explants of tomato variety Nadir

based on the root explants (F = 163.00, P < 0.0001). This result implies that the two genotypes have different abilities to regenerate roots from explants. Nadir generated an average of 20.00 roots per explant, but Naqeeb produced an average of 22.00 roots per explant, according to further analysis using the Least Significant Difference (LSD) test. Naqeeb and Nadir were placed in groups A and B, respectively, based on the genetic classification into two separate groups. There was a statistically significant difference (SE = 0.8165, T-value = 2.776) between the two genotypes.

4 | DISCUSSION

Seeds of each cultivar of tomato (*Lycopercsicon esculentum*) i.e. "Naqeeb" and "Nadir" were inoculated on basal MS medium. Naqeeb var. showed a germination rate of 87.3%, whereas, Nadir resulted in 85.5% germination. Around 9% and 8.2% seeds of "Naqeeb" and "Nadir" respectively failed to germination and 3.7% seeds of Naqeeb and 6.3% seeds of Nadir were found contaminated as shown in Table: 3. This indicated that both genotypes had high germination rate under same experimental conditions.

Cotyledons, hypocotyls, leaf discs and roots from both varieties were used as ex-plants. 96 explants from each variety of tomato were cultured on MS media supplemented with different concentrations of NAA/6-benzyl aminopurine as given in Table: 1. The callus growth was affected considerably by different levels of hormones. Both varieties showed different regeneration efficiencies but some overall findings noted are: Media without any hormone showed no Callogenesis. T9 produced highest callus induction from cotyledon (72.2%) and leaf discs (77.8%) for Naqeeb. Whereas, T8 showed highest callus formation from roots (59.7%) and T6 was proved best for callus from hypocotyls (64.3%) for cultivar Naqeeb. T1 resulted in least callogenesis for all ex-plants (Fig: 2). The same pattern of callus induction was recorded in the case of Nadir variety (Fig: 2) which indicates T9 for cotyledons (76.7%) and leaf discs (64.1%); T8 best for roots (57.7%) and on T6 hypocotyls produced maximum callus i.e. (69.2%). This comparison predicted an overall superiority of Tomato variety Naqeeb.

In the next step of the experiment calli from all explants were sub-cultured and shifted to regeneration medium containing basal MS medium alongwith various levels of Zeatin and IAA (Table: 2). Many scientists used indole acetic acid and Zeatin alongwtih 6-benzyl aminopurine and Kinetin for Callogenesis and regeneration. Likewise (Afroz et al. 2009) used GA3 in conducting an experiment for three varities of tomato to achieve a quick, high rate of regeneration. Regeneration period was reduced to half in all varieties due to GA3.

In the current research all the calli instead of moving towards shoot induction started to regenerate roots. Out of all 3 regeneration media, R2 and R3 proved better for root induction as compared to R1 media containing Zeatin: IAA as 1.0: 0.1mg/L and 1.0mg/L: 1 mg/L respectively. (Table 4). Results also indicated that the increased levels of Zeatin reduce the rooting efficiency (Costa et al. 2000). Through research indicated that the cotyledonary calli from two varieties of tomato ‘IPA-5’ and ‘IPA-6’ presented 97% and 80% respectively shoot regeneration on MS media supplemented with 1mg/L Zeatin and 0.1mg/L IAA. But in this research same concentrations of both hormones used for all calli of two varieties of tomato Naqeeb & Nadir. Research done by (Gubis et al. 2004), conclusion was given that MS basal media along with 1 mg /l of Zeatin and 0.1 mg/ l of IAA was proved as best regeneration medium for regeneration from hypocotyls of tomato *(Lycopersicon esculentum* Mill.) that showed high regeneration rate. But some varieties used in this research did not show good regeneration rate, which depicts that regeneration relied on genetic makeup. In an experiment 69% shooting regeneration frequency

for tomato cultivar money maker on MS medium concentrated along with 1mg/L of Zeatin and 1mg/L IAA. It was also reported that leaf discs did not show any regeneration on this media. At the end of the research, all the tomato shoots obtained on shoot induction media were to be transferred to rooting media comprising 0.5 MS medium

Table 4: Overall Root Induction Frequency from all explants

Variety	R1			R2			R3		
	Hypocotyl	Root	Cotyledon	Hypocotyl	Root	Cotyledon	Hypocotyl	Root	Cotyledon
Naqeeb	29.556	8.444	36.222	29.556	27.333	43.889	38.889	24.778	43.111
Nadir	31.467	10.133	35.467	35.467	32.800	42.667	42.667	26.733	45.733

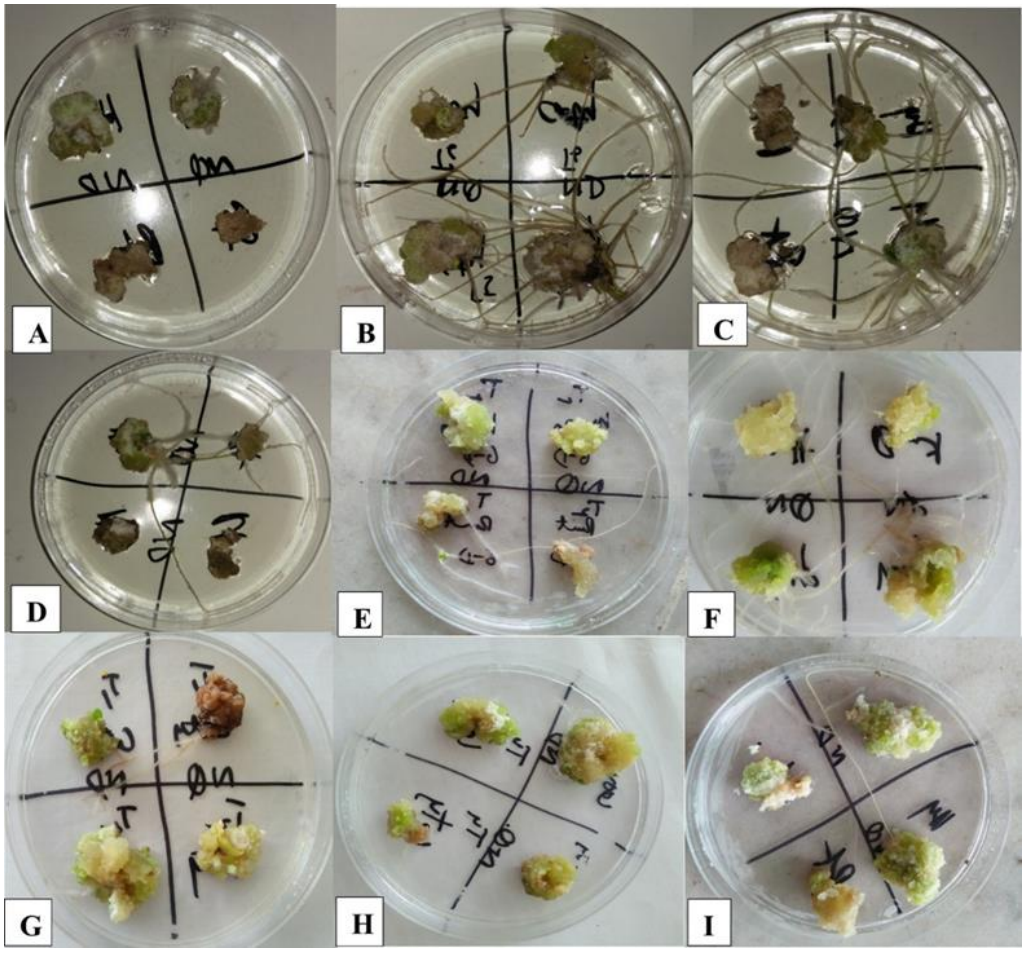

Fig. 3: Rate of Root induction of different explant using different treatments A) Root Induction from R1: T5; hypocotyl; root B) Root Induction from R1; T5; Cotyledon C) Root Induction from R1; T8; Hypo, root D) Root Induction from R1; T4 Hypo, root E) Root Induction from R1; T2; hypo, root F) Root Induction from R2, T2, hypo, root G) Root Induction from R2, T1, T2 cotyledon H) Root Induction from R3, T 3, 4, cotyledon I) Root Induction from R3; T6, hypo, root

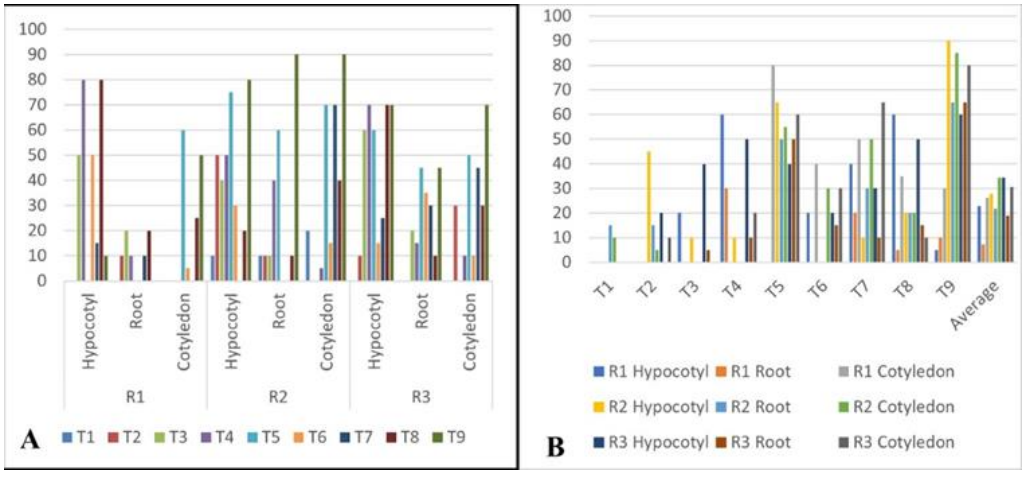

Fig. 4: (a) Root Induction frequencies in tomato variety Naqeeb. (b) Root Induction frequencies in tomato variety Nadir

fortified with 0.2mg/L IBA and 0.25 mg/L 6-benzyl aminopurine for root initiation. Comparison for both root induction frequency of naqib and nadir variety is shown in Fig. 4. But because of non induction of shoots and induction of roots instead changed the complete scenario. The possible factors for such repression may

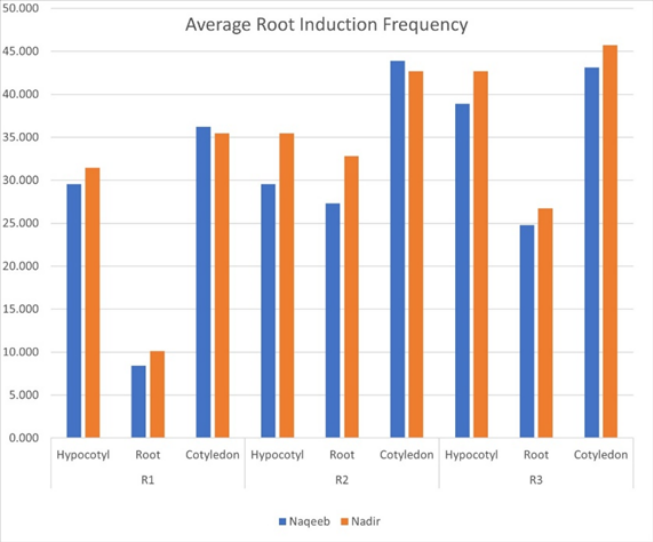

Fig. 5: Overall Root Induction Frequency from all explants

be certain environmental conditions, hormonal conditions for varieties used in this research, age of calli used. There may be a chance of genetic makeup of these varieties which is not expressed by hormones that used. Hope that future researcher may find out the possible hormonal combinations or regeneration protocol for best regeneration of Nadir and Naqeeb varieties.

Conclusion

Overall, variety and explant type both significantly affect root regeneration, with Naqeeb hypocotyl explants offering the best results. The variation in regeneration between the explant types highlights that hypocotyls are the most responsive tissues, likely due to their undifferentiated nature and higher physiological plasticity compared to root and cotyledon tissues. These insights provide a solid foundation for further research on optimizing tomato regeneration techniques for improved efficiency in plant propagation and breeding.

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