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Protocol for Anther Callus Formation for Selected Genotypes of Tomato (*Solanum lycopersicum* L.)

T.M.S.H. Thennakoon[1], H.M.P.S. Kumari[2] and P.A. Weerasinghe[1]

[1]*Department of Plant Sciences, Faculty of Agriculture, Rajarata University of Sri Lanka, Anuradhapura, Sri Lanka.*

[2]*Horticultural Crops Research and Development Institute, Gannoruwa, Peradeniya, Sri Lanka.*

Correspondence Author
P. A. Weerasinghe
Department of Plant Sciences, Faculty of Agriculture,
Rajarata University of Sri Lanka, Puliyankulama 50000 Anuradhapura,
Sri Lanka.

aruni500@yahoo.com
aruniw@agri.rjt.ac.lk

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Abstract: *Anther culture technology is one of the most rapid methods that can be used to accelerate plant breeding programmes by producing double haploids. Therefore, this study aimed to develop an effective callus induction medium for selected tomato genotypes. Four treatments with varying Benzyl Amino Purine (BAP) concentrations (0, 1, 2, and 3 mg L^{-1}) were tested, each containing 2 mg L^{-1} Kinetin and 1 mg L^{-1} Naphthalin Acetic Acid (NAA) on full-strength MS medium. Flower buds from varieties NO-312, HT 05, Bathiya, L-33, and 12-561 were used. After preparation and surface sterilization, the anthers were incubated at 25°C in the dark for 14 days to induce callus formation. Results showed that the highest callus formation (42.08±14.2%) was in the 0 mg L^{-1} BAP treatment for variety NO-312. The highest callus greening (14.28±14.2%) was in the 0 mg L^{-1} BAP treatment for variety HT 05. Thus, NO-312 was the best for callus formation and HT 05 for callus greening. The study indicates that the absence of BAP positively affects callus formation and greening in these tomato genotypes, aiding in refining anther culture methods for faster plant breeding.*

I. INTRODUCTION

All over the world, tomato (*Solanum lycopersicum*) holds a preeminent status as one of the most vital edible crops. Its significance is underscored by its valuable agronomic characteristics, nutritional profile, and the higher demand for this marketable product. Consequently, tomato plants have garnered considerable attention, not only from producers but also from researchers who are exploring both conventional and unconventional

breeding practices [1]. Of the various regeneration systems available, the biotechnological approach involving *in vitro* androgenesis has emerged as a subject of great interest [2].

The initial method of anther culture, pioneered by Guha and Maheshwari in 1964 and 1966 [3], has undergone several adaptations by different researchers. These modifications have proven fruitful in numerous plant species, where positive outcomes have been achieved using isolated microspores or pollen grains in addition to inflorescence cultures [4].

The anther culture technique finds application in generating homozygous lines essential for hybrid seed production, with a focus on the Solanaceae and Cruciferae plant families. However, it's worth noting that the ability to produce haploids within the Solanaceae family is relatively limited. Hence, there is a significant emphasis on enhancing anther culture technology to cater to diverse genotypes of tomatoes [5].

The anticipated tomato consumption in Sri Lanka is expected to reach 98,000 metric tons by the year 2026, reflecting a modest annual growth rate of 0.8% compared to the 93,000 metric tons recorded in 2021. This foretells a substantial increase in tomato demand. However, relying solely on traditional breeding methods may present challenges in meeting this heightened demand. Anther culture technology can be employed to develop haploid breeding lines in tomatoes, avoiding the drawbacks of diminished fitness and genetic diversity associated with lengthy breeding cycles[6] . Utilizing the most suitable medium in anther culture, encompassing effective callus induction and regeneration media can expedite the plant breeding program [7].

Hence, this study aims to identify the optimal callus induction medium and varieties for selected tomato genotypes. The establishment of the best callus induction medium is expected to enhance the production of diverse tomato varieties for planting materials.

II. Materials and Methods

Widely grown commercial tomato varieties such as Bathiya and four inbreed lines 12-561, HT- 05, NO 312 and L-33 were selected for the study as mentioned in Table 1. Tomato plants were cultivated at a plant house in Horticultural Crop Research and Development Institute (HORDI) Gannaoruwa, Peradeniya, Sri Lanka.

Table 1. Varieties and inbred lines for the doner plant.

Variety/ Breeding line	Description
Bathiya	Released by the Department of Agriculture, Sri Lanka
12-561	Breeding Line
HT-05	Breeding Line
NO-312	Breeding Line
L 33	Breeding Line

Collection of flower buds

Floral buds were collected from parent plants between 3 and 4 pm to isolate anthers and identify the optimal developmental stage for inducing anther callus (Fig 1). Care was taken to preserve sections of the peduncle to reduce infection risk. The selected buds had crown petals nearly identical to or slightly longer (about 1 mm) than the calyx. After collection, the buds were cooled at 4°C for 12 hours.

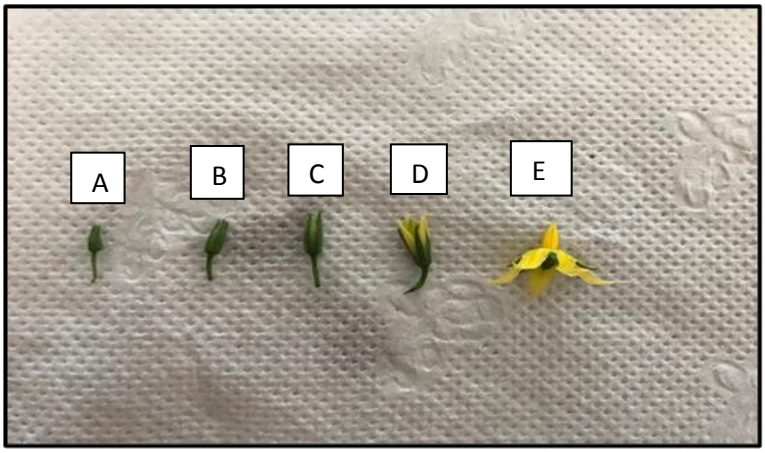

Figure 1. C- The optimal stage for collecting tomato buds

Pre-Surface Sterilization of Flower Buds

Before taking flower buds to the laboratory, they were pre-sterilized by washing them with dishwashing liquid under running water for 20 minutes in the evening. The buds were then covered with aluminium foil and refrigerated overnight. On the day of culturing, the buds were further sterilized by submerging them in 70% ethyl alcohol for 1 minute. This thorough sterilization reduced the risk of contamination from surface pathogens.

Surface Sterilization of Flower Buds

The procedure was conducted in a laminar airflow cabinet to maintain aseptic conditions. Flower buds were first sterilized in a 40% Clorox (bleach) solution for 15 minutes, then rinsed with distilled water to remove any Clorox residues. The cleaned buds were transferred to a pre-sterilized, oven-heated Petri dish.

Preparation of Stock Solutions

The MS medium (Murashige & Skoog, 1962) was meticulously prepared by creating separate stock solutions for macro and micronutrients, FeNa EDTA, and vitamins. These stock solutions were poured into autoclaved reagent bottles for sterility and stored in a refrigerator. This precise preparation ensured the uniform composition of the MS medium for the experiment.

Treatment Composition

As shown in Table 2, a full-strength MS solid medium was prepared with combinations of NAA (1 mg L^{-1}), Kinetin (2 mg L^{-1}), and varying concentrations of BAP (0, 1, 2, 3 mg L^{-1})

Table 2. MS solid medium with NAA+ Kinetin + BAP on callus induction of tomato.

Treatment	Composition of the medium
T1	MS media + NAA (1 mg L^{-1}) + Kinetin (2 mg L^{-1}) + BAP (0 mg L^{-1})
T2	MS media + NAA (1 mg L^{-1}) + Kinetin (2 mg L^{-1}) + BAP (1 mg L^{-1})
T3	MS media + NAA (1 mg L^{-1}) + Kinetin (2 mg L^{-1}) + BAP (2 mg L^{-1})
T4	MS media + NAA (1 mg L^{-1}) + Kinetin (2 mg L^{-1}) + BAP (3 mg L^{-1})

In Vitro Culturing of Anthers

The operations were conducted in a laminar airflow cabinet under aseptic conditions. With the help of forceps and scalpel, the petals of the flower buds were removed and anthers were excised. Then the anthers were placed on pre-prepared callus induction medium bottles each containing a single variety.

Culture Environment for Callus Induction

During initial callus formation, the cultures were kept in the dark at 25-28°C. Once callus initiation occurred, the bottles were exposed to 2000 lux light intensity for a 16-hour photoperiod.

Collection of Experimental Data and Data Analysis

The experiment was designed in a Completely Randomized Design (CRD) with 3 treatments and 1 control. For each of the five varieties, 3 replicates per treatment were included totalling 60 culture bottles. Data collection was done weekly, recording swollen anthers, callus quantity, colour, appearance, shape, and contamination. Analysis was conducted using R software (version 4.3.0), with effects of treatment and variety examined via two-way ANOVA, and mean differences analyzed through the LSD test.

III. Results and Discussion

Callus Formation

Formation of callus initiated within 14 days after culturing as shown in Table 3.

Table 3. Callus formation percentage in different varieties in different treatments.

	HT 05	Bathiya	NO – 312	L 33	12 – 561
T1 – BAP 0 mg L^{-1}	15.13^{ab}	21.86^{ab}	42.8^{a}	28.13^{ab}	23.03^{ab}
T2 – BAP 1 mg L^{-1}	30^{ab}	13.7^{ab}	17.03^{ab}	9.06^{b}	6.5^{ab}
T3 – BAP 2 mg L^{-1}	26.53^{ab}	26.66^{ab}	20^{ab}	3.33^{b}	15.46^{ab}
T4 – BAP 3 mg L^{-1}	10^{b}	9.36^{b}	11.1^{b}	3.33^{b}	25^{ab}

Figure 2. The callus formation in variety NO – 312 in treatment 01

It has been reported [8] that a balance between auxin and cytokinin in an artificial medium determines the callogenesis, therefore, MS media supplemented with different concentrations of auxins and cytokinins were used for callus induction. Also for some members of Solanaceae family, the inclusion in the culture medium of an auxin alone, or in combination with a cytokinin, frequently induces the formation of pollen calli [9].

The present investigation revealed (Table 3) that the percentage of callus formation has a significant effect ($p<0.05$). The maximum callus formation percentage (42.8 ±14.2) was observed in the treatment with 0.0 mg L^{-1} BAP from variety NO – 312 (Fig 2), while the minimum percentage (3.33±5.7) was noted in the treatment with 3 mg L^{-1} and 4 mg L^{-1} BAP from variety L 33. Similar observations were reported by [10] where explants of *Sauropus endogynous* were cultured in low concentrations of cytokinin hormone which alone did not produce any callus when compared with a medium supplemented with auxin. However, It was found [11] that a high concentration of BAP combination with low concentration of NAA frequently produced callus in a shorter period of time than that of other hormonal combinations.

According to Table 4, variety vise comparison had a significant effect on anther callus formation. According to the LSD test, there was a considerable significant difference among varieties. The highest mean callus formation (22.73±15.38) was observed in variety NO – 312 and followed by HT 05, Bathiya and 12-561 (Table 4). The lowest callus formation was observed in L 33 (11.04±13.26). And also [12] similar investigation showed callus formation varying based on treatments and genotype of the cultivars.

Table 4. Mean callus formation in different tomato varieties.

Variety	Mean Callus Formation
HT 05	20.52a
Bathiya	17.9ab
NO – 312	22.73ab
L 33	11.04ab

According to Table 5, the highest mean callus formation percentage (26.27±4.51) was observed in treatment 01 while the lowest mean callus formation percentage (10.09±8.46) was in treatment 04. A similar study [10] reported that among various MS media with 1 mg L^{-1} NAA and 2 mg L^{-1} Kinetin was found to be the best for callus induction. However, in this study, different genotypes responded differently in the media as shown in Table 5.

In this study, the highest callus formation was given by treatment 1 which have had combined higher auxin and lower cytokinin hormone concentrations. A similar study was done by [13] on media that used combined auxin and cytokinin at a ratio with higher auxin and lower cytokinin improved callus formation.

Table 5. Variation of mean callus formation within treatments.

Treatment	Mean Callus Formation
T1 - BAP 0 mg L^{-1}	26.27^{a}
T2 - BAP 1 mg L^{-1}	17.5^{ab}
T3 - BAP 2 mg L^{-1}	18.4^{ab}
T4 - BAP 3 mg L^{-1}	10.09^{b}

Callus Greening

In this experiment, some of the calli turned green after 2 to 8 weeks after culturing. With the influence of light, the crystalline calli were turned into a soft pale green colour. Then the middle top region of the callus became white colour. Subsequently, the whole callus gradually turned green colour.

According to similar study showed that the callus colour indicates the presence of chlorophyll in the tissue. The greener colour of the callus means more chlorophyll content. A light or white colour calli can indicate that the

callus condition is still relatively good [2].

Table 6. Callus greening percentage of different varieties in different treatments.

Treatment	HT 05	Bathiya	NO – 312	L 33	12-561
T1 - BAP 0 mg L^{-1}	12.22[a]	8.88[a]	14.28[a]	10.74[a]	9.7[a]
T2 - BAP 1 mg L^{-1}	6.66[a]	6.66[a]	6.66[a]	3.03[a]	10.37[a]
T3 - BAP 2 mg L^{-1}	8.88[a]	5.77[a]	5.8[a]	3.33[a]	5.22[a]
T4 - BAP 3 mg L^{-1}	10[a]	6.36[a]	5.55[a]	0	6.66[a]

According to Table 6, the maximum percentage (14.28± 14.28) of callus greening, was observed in the 0 mg L^{-1} BAP treatment in variety NO - 312, while the minimum greening percentage (0%) was observed in the 3 mg L^{-1} BAP treatment for variety L 33.

Table 7. Variation of mean callus greening within treatments.

Treatment	Mean Callus Greening
T1 - BAP 0 mg L^{-1}	11.16[a]
T2 - BAP 1 mg L^{-1}	6.68[a]
T3 - BAP 2 mg L^{-1}	6.11[a]
T4 - BAP 3 mg L^{-1}	5.71[a]

In this study, callus greening didn't have a significant effect among treatments. According to the LSD test, the highest mean callus greening percentage (11.16 ±8.01) was obtained in treatment 01 with 0 mg L^{-1} BAP while the lowest mean callus greening percentage (5.71±4.08) was given by treatment 04 with BAP 3 mg L^{-1} (Table 7). However, in the study [14] revealed that, treatment which have different BAP and NAA concentration ratio produce pale yellow colour calli while the medium supplemented with 1.0 mgL^{-1} BAP and 1.0 mg L^{-1} NAA (same ratio of BAP and NAA) produce more green colour calli.

Table 8. Mean callus greening amoung varieties.

Variety	Mean Callus Greening
HT 05	9.43_a
Bathiya	7.14[a]
NO – 312	8.07[a]
L 33	4.27[a]
12-561	8.15[a]

Callus greening did not have a significant effect among varieties (Table 8). According to the LSD test, five different varieties showed similar grouping (a). However, the highest mean value of callus greening percentage (9.44±5.09) was in variety HT 05. The lowest mean callus greening percentage (4.27±7.11) was observed in variety L 33. When a callus has more greening, it has more chlorophyll content. So, the HT 05 variety calli have a higher possibility of undergoing organogenesis and producing a whole plant[2] . Also, other varieties (Bathiya, NO – 312, and 12-561) were given a considerable amount of callus greening.

IV. Conclusion

It can be concluded that treatment 01 with 0 mg L^{-1} BAP concentration was the best treatment for callus formation and greening. And also, the highest mean callus formation was given by variety NO – 312. Thus, NO – 312 was the best variety to practice anther culture among the tested five genotypes. Though it is not significant, HT 05 showed the best callus greening.

This study reveals that the medium which not supplemented with BAP had positive effects on callus formation and greening for selected genotypes of tomato. This information helps refine anther culture methods for faster plant breeding.

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