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Effect of Cytokinins Concentration and Types for Shoot Induction and Multiplication Experiments of in Vitro Propagation of Black Pepper (Piper Nigrum L.) from Nodal Culture

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Abstract **– Black pepper belongs to the family Piperaceae. It is a perennial woody climbing tree. In Ethiopia, black pepper, known as ''Kundo berbere' or 'Yebahir kimem'. Lack of a steady supply of quality planting material is one of the bottlenecks for the exploitation of the export potential of black pepper. Thus use of micro propagation is suggested to alleviate the problem of shortage of planting materials. In vitro seed germination and multiplication of nodal explants to regenerate shoots is preferred because it minimizes sterilization cost. So far there is no available efficient protocol optimization on in vitro propagation of black pepper in Ethiopia. Therefore, the objectives of the present study were to develop a protocol for an efficient in vitro propagation from nodal culture of black pepper. Using seeds as explants source shoot initiation, multiplication were carried out using CRD design in factorial arrangement at jimma Agricultural Biotechnology Laboratory. Five levels of both cytokinins (benzyl adenine, 0-4mg/l) and (Thidiazuron, 0-0.85mg/l) supplemented in full MS medium were used for shoot induction and multiplication experiments.. Nodal explants isolated from in vitro germinated seedlings (50-days-old) were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of TDZ (0, 0.25, 0.45, 0.65, &0.85 mg/l) and BA (0, 1, 2, 3 & 4mg/l) accordingly. Data were subjected to ANOVA and significant different means were separated using LSD test. In shoot induction and multiplication experiments 4mg/l BA alone supplemented with full MS medium showed the shortest days to shoot induction (38.67), the maximum length of shoot (3.16cm), the highest number of shoot per explant (9.51), but 0mg/l of TDZ gave the highest number of leaves per explants (3.33). The findings in the present study would be helpful for large-scale mass propagation of black pepper using this important and efficient protocol.**

Keywords **– Cytokinins, Explants, Nodal Culture, Shoot Induction, Shoot Multiplication**.

I. INTRODUCTION

Black pepper (*Piper nigrum* L.) belongs to the family Piperaceae. It is a perennialwoody climbing tree. It is native to India, Indonesia, Malaysia, South America and West Indies but is also widely cultivated in the tropical regions. Black pepper is a universal table condiment used to flavor all types of cuisines worldwide. It is christened as the 'King of Spices' [7], [14].

In Ethiopia, black pepper is known as ''*Kundo berbere'* or '*Yebahir kimem'*, it used all over the country as a seasoning during food preparation. Ethiopia had exclusively been importing black pepper to cover the national demand; this in turn has made the spice unaffordable to the vast majority that dried fruits of *Schinus molle* were widely used as its cheaper substitute [2].

In the shoot multiplication stage, each explant has expanded into a cluster of micro shoots arising from a basal mass of callus-like tissue. This structure is divided into separate micro shoots, which are transplanted into new culture medium. The kind of medium used depends on the plant species, growth culture and type of culture [3].

There is generally a minimum mass of tissue culture that is necessary to produce uniform and rapid multiplication for the next transfer. The number of micro shoots produced ranges from 5 to 25 or more depending upon the species cultured and conditions of culture. Multiplication may be repeated several times to increase the supply of material to a predetermined level for subsequent rooting and transplanting. Sometimes micro shoots deteriorate with time, lose leaves, fail to grow, develop tip-burn and go dormant or lose potentiality to regenerate due to the lack of stabilization of the culture medium. Stabilization involves a change in growth characteristics from unpredictable and often abnormal shoots development at first to a predictable growth pattern [4].

The difficulty in stabilization appears to be associated with the phase of the explants. Most annuals and herbaceous plants stabilized quickly within a few subcultures whereas woody plants invariable, require much longer and in some may never achieved (various reports indicated that, the combination of phytohormones often determine the course of morphogenesis, for instance, shoot organogenesis or embryogenesis [3].

The type and concentration of cytokinins used in the shoot multiplication depends upon the type and kind of genotypes and plant species. For instance, in some plant species the reduction of shoot height caused by TDZ could be attributed to its strong cytokinins-like activity. TDZ could be show to stimulate shoot proliferation than shoot elongation [4], [6].

In vitro culture offers improvements over traditional vegetative propagation because of faster rate of

multiplication. Nowadays the world spice (black pepper) demand is highly increasing; conventional method of propagation of black pepper will not meet the high demand for planting materials of the spice. It is difficult to achieve this huge goal by conventional propagation methods, so it is necessary to propagate them though tissue culture. But there is no any work done to propagate black pepper though tissue culture in Ethiopia and to do that optimization of *in vitro* propagation protocol for the propagation of this crop to utilize is a must, therefore the objectives of this piece of work is to determine effect of cytokinins concentration and types for shoot induction and multiplication experiments.

II. Materials and Methods

2.1. Description of the Experimental Area

The experiment was conducted during 2015/16 at the Tissue Culture Laboratory of Plant Biotechnology Division in Jimma Agricultural Research Center (JARC), South Western Ethiopia. The center is located 361 km far from Addis Ababa in Southwest of Ethiopia at $7^0 40^{'}$ 15''N latitude and $36^0$49'33"E longitude and at an altitude of 1750 m.a.s.l. (EARO, 2000).

2.2. Sources of Experimental Material

Ripe seeds were collected from four years old healthy plants of piper nigrum L. cultivar Tato/380 from Teppi national spices center in October 2016 cropping season.

2.3. Experimental Procedure

2.3.1. Culture Media Preparation

In all cases, culture media were prepared by taking the recommended amounts of MS stock solutions supplemented with 3% (w/v) sucrose as a carbon source, and 0.7% (w/v) agar (for shoot multiplication) and 0.7% (w/v) agar (for root induction) as solidifying agents. In each experiment, the desired concentrations and types of PGR were added before autoclaving and 40 ml of the respective medium was poured into 350 ml jams jar and covered with a cap for both shoot multiplication and root induction experimental phases. After mixing up all media components together with the combined PGR and adjusting the volume, the pH of the culture medium was adjusted to 5.80 with either 1% N HCl or 1% N NaOH. Later, the respective solidifying agent (agar) was added into the medium. Medium was autoclaved at 15 pounds per square inch (psi) i.e. 121^0C for 20 minutes. Autoclaved media was allowed to cool in sterile environment after which it was ready for use. Finally, all the autoclaved culture media were retained in the media room for a maximum of three days prior to use.

2.3.2. Effect of Cytokinins on Shoot Induction and Multiplication

The germinated seedling produced was maintained in vitro and was used as a source of contamination free explants for further experiments. Nodal segments of (1.5 cm)length was excised and implanted to basal FMS medium supplemented with various combination of cytokinins Benzyl-adenine (0-4mg/l) and Thidiazuron (0-0.85mg/l) for shoot induction and multiplication experiment.

In order to identify the best combinations of cytokinins concentrations for shoot induction and multiplication, five levels of TDZ (0, 0.25, 0.45, 0.65, and 0.85mg/l) and BA (0, 1, 2, 3, and 4 mg/l) were combined. In each experimental unit, three nodal segments (1.5 cm) were cultured per jar/replication with a total observation unit of nine explants per treatment. All studies were repeated two times, though only data from the last one was used for statistical analysis. Cultures were incubated in the culture room at a constant temperature of 25 ± 2^0C and relative humidity of 50-60%, under cool white fluorescent light of 28 µmol/m2 (1500-2000 lux) photosynthetic photon flux density with 16 hour per day photoperiod for six weeks. After six weeks, data were recorded from individual emerged new shoots obtained from each cultured shoots and subsequently cultured on free hormone media.

After taking of data of the shoot growth parameters, the original shoots were transferred to fresh treatment medium for further shoot multiplication. Over a period of six weeks.

2.4. Data Recorded

- Number of days to shoot induction: The number of days to shoot induction was recorded after six weeks of nodal culture and their means was taken for analysis. The number of days taken to show initial differentiation of shoot from the date of inoculation of different explants was recorded and was expressed as mean number of days.
- Number of shoots: The total number of new emerged Shootlets were counted per explant and divided by the total number of explants initially cultured and their means were taken for analysis.
- Number of leaves: The total number of leaves emerged from each Shootlets were obtained by counting the representative leaves and then divided by the total number of Shootlets per each replication of treatment combinations and the mean number of leaves per shoot for each treatment combination were taken
- Shoot length (cm): The average shoot length was measured by taking three representative Shootlets per replication in the laminar hood to prevent Shootlets/plantlets from contamination. The shoot length was measured from crown to the tip of the Shootlets at the time of subculture and the average length was expressed in centimeters.

2.5. Data Analysis

The Data were subjected to analysis of variance (ANOVA) using the SAS software Packages (version 9.2) and significant differences among mean values was compared using Least Significant Difference (LSD) at 5% of probability level.

III. Results and Discussion

3.1. Combined Effect of Different Concentration of TDZ and BA on Shoot Induction and Multiplication Stage.

In the multiplication stage, the ANOVA showed significant effects on the type and concentration of cytokinins. In this study, the analysis of variance revealed that the different cytokinins concentrations of BA and TDZ combinations tested had no significance difference on mean

number of leaves per shoots, and mean shoot length (Table 2). However, the effect of these cytokinins combinations on the number of days to shoot induction and mean number of shoot of all treatments tested were found to be statistically highly significant (Table 1 and Figure 2).

Table 1. Mean and probabilities of the independent effect of TDZ and BA on black pepper shoot induction and multiplication

Treatments	Mean(main effect)	
	MNLPS	MSL
TDZ(mg/l)		
0 mg/l TDZ	3.33^{A}	3.13^{A}
0.25mg/lTDZ	2.10^{D}	2.62^{B}
0.45mg/lTDZ	2.32^{C}	2.63^{B}
0.65mg/lTDZ	2.76^{B}	2.75^{A}
0.85mg/lTDZ	2.69^{B}	2.71^{A}
BA (mg/l)		
0 mg/l BA	1.62^{C}	2.10^{D}
1 mg/l BA	2.71^{B}	2.93^{B}
2 mg/l BA	3.13^{A}	2.91^{B}
3 mg/l BA	3.16^{A}	2.75^{C}
4 mg/l BA	3.07^{A}	3.16^{A}
PROB		
TDZ	0.003^{**}	0.0683^{ns}
BA	$<.0001^{**}$	$<0.0001^{**}$
(TDZ*BA)	0.8296^{ns}	0.3106^{ns}
CV (%)	14.42	19.13

Note that: where, ** = highly significant difference at 1% probability level and * significance at 5%probability level (LSD). Means within a column followed by the same letter are not significantly different at P < 0.05 level of significance (LSD). Where, No- number, Prob. - probability, in induction and NS-non significance. MNLPS: mean no of leaves per shoot, MSL: mean shoot length. BA = N-Benzyl adenine. TDZ = Thidiazuron

Table 2. Combined Effects of TDZ and BA in shoot induction and multiplication

TDZ &BA(mg/l)		MNDSI	MNOS
0	0	50.33 ± 0.67^{C}	6.29 ± 0.32^{G}
0	1	45.67 ± 0.4^{H}	8.21 ± 0.11^{C}
0	2	44.33 ± 0.46^{I}	8.69 ± 0.41^{C}
0	3	41.33 ± 1.03^{J}	9.11 ± 0.11^{B}
0	4	38.67 ± 0.58^{K}	9.51 ± 0.06^{A}
0.25	0	52.33 ± 1.18^{B}	7.13 ± 0.25^{E}
0.25	1	45.33 ± 2.24^{H}	6.81 ± 0.28^{F}
0.25	2	48.67 ± 0.32^{E}	7.76 ± 1.46^{D}
0.25	3	46.67 ± 2.67^{G}	7.66 ± 1.62^{D}
0.25	4	45.33 ± 2.34^{H}	7.13 ± 0.50^{E}
0.45	0	51.33 ± 1.56^{B}	7.08 ± 0.34^{E}
0.45	1	44.67 ± 0.73^{I}	6.36 ± 0.79^{G}
0.45	2	45.67 ± 1.26^{H}	7.79 ± 0.32^{D}
0.45	3	46.67 ± 3.76^{G}	7.12 ± 1.15^{E}
0.45	4	47.33 ± 2.55^{F}	6.53 ± 0.64^{F}
0.65	0	50.33 ± 0.91^{C}	7.43 ± 0.32^{D}
0.65	1	47.05 ± 1.32^{F}	6.73 ± 0.45^{F}
0.65	2	49.80 ± 0.70^{D}	7.69 ± 0.65^{D}
0.65	3	47.33 ± 0.64^{F}	7.66 ± 0.14^{D}
0.65	4	47.67 ± 1.47^{F}	6.95 ± 0.66^{F}
0.85	0	53.33 ± 1.32^{A}	6.69 ± 0.41^{F}
0.85	1	50.33 ± 0.37^{C}	7.98 ± 1.42^{D}
0.85	2	51.33 ± 0.62^{AB}	7.50 ± 0.250^{D}
0.85	3	49.33 ± 2.68^{D}	7.00 ± 0.55^{E}
0.85	4	50.33 ± 1.72^{C}	6.83 ± 0.25^{F}
MEAN		47.56	7.42
CV (%)		3.38	9.31

Note that: Means the same letter are not significantly different at P < 0.05 level of significance. Where, MNDSI = mean no. of days to shoot induction, MNOS=mean no of shoot per explant. BA = N-Benzyl adenine. TDZ = Thidiazuron

3.1.1. Mean Number of Days to Shoot Induction

The combined effect of TDZ + BA, TDZ and BA alone highly significant effect on mean number of days to shoot induction (Table3). The least number of days to shoot induction was recorded on the treatment combination of 0+4mg/l of TDZ + BA (38.67). Even though the combined and independent effects of hormone combination were highly significant, BA alone gave better response to shorten the number of days to shoot induction.

The highest number of days to shoot induction was recorded from apply of 0.85 + 0mg/l TDZ + BA (53.33). This shows that as the concentration of TDZ increase from 0 to 0.85 the number of days to shoot induction also increases. Therefore to induce shoot within short period of time it is better to decrease the concentration of TDZ. The lowest response of number of shoots was noticed in BA 2.0 mg/l while the highest response was observed in medium supplemented with BA 5.0 mg/l in some varieties of spices. The ability of BA to induce multiple shoots was also reported by different researchers. Perennial spice plants are highly responsive to BA treatments and most cultured perennial species produce robust, well-formed shoots suitable for further shoot proliferation [8].

3.1.2. Number of Shoots

BA, TDZ, BA and TDZ in combination had highly significant effect on mean number of shoots per explant (Table 3). The highest number of shoot was recorded on 4+0mg/l BA+TDZ, which was (9.51), followed by 3+0 mg/l BA and TDZ (9.11). The lowest number of shoot was recorded on PGR free medium (6.29), followed by 0.45 + 1mg/l TDZ + BA (6.36). [9] found that a concentration of 5.0 mg/l BA was the best for shoot multiplication for Curcuma demostica. In this study, 4mg/l BA was best for shoot proliferation of P. nigrum. [11] Reported that both TDZ and BA were individually more effective in shoot formation on Jatropha curca. Similarly [5] reported that 0.5mg/l TDZ showed best shoot regeneration on curcuma demostica. High concentration of TDZ increases regeneration of shoots in perennial spices. There are two hypotheses in this regard, the first hypothesis expresses that the hormone induces regeneration by direct stimulation of tissue; while the second one state that TDZ stimulates indigenous cytokinins and thereby facilitates shoot regeneration as reported by [12] reported

3.1.3. Shoot Length (cm)

BA alone were highly significantly affected the shoot length with $P \leq 0.001$, but TDZ was not significant ($p = 0.0683$) on mean shoot length. Hence 0 mg/l BA was observed a minimum mean shoot length (2.10 cm). The maximum shoot length was observed in BA 4mg/l (3.16

cm), followed by BA 1mg/l (2.93 cm). As the concentration of BA increase from 0-4mg/l the shoot proliferation rate also increase. Nordstrom et al, (2004) reported that higher BA concentrations always promoted more shoot development (shoot length) in T officinal. And [13] reported that maximum number of shoot was recorded on MS medium supplemented in 2mg/l BA for pepper nigrum. [1] reported that BA was effective for the multiplication of in vitro raised plants of P. longum, Piper betle and Piper nigrum

3.1.4. Number of Leaves

The interaction effect of both TDZ and BA (TDZ*BA) were not highly significant (P = 0.8296) on mean number of leaves per shoot. The number of leaves was highly significant (P ≤ 0.01) on BA concentration and significant (0.0166) on TDZ concentration (Table 2) The maximum number of leaves was found on 0 mg/l of TDZ (3.33), and the minimum number of leaves (2.10) was recorded on 0.25mg/l TDZ. The maximum number of leaves (3.16) on BA concentration was recorded on 3mg/l of BA, but the minimum number of leaves (1.62) was recorded on 0mg/l of BA. Increasing the amount of cytokinins like BA up to 5 mg/l gave as the maximum number of leaves per shoot as reported by [13].

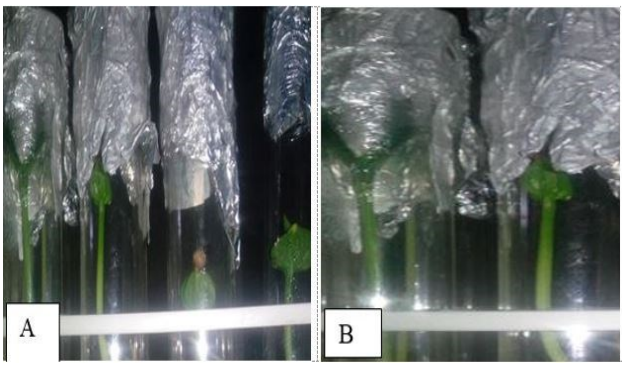

Fig. 2. Effect of BA and TDZ on *in vitro* shoot induction and multiplication of black pepper
A. Shoot induced in 3 mg/l BA + fullMS medium
B. Shoot proliferated in 4 + 0mg/l BA + TDZ

IV. Summary, Conclusions and Recommendations

4.1. Summary and Conclusions

While tissue culture based rapid multiplication proved to be promising in different species of plants including black pepper, optimizing efficient and reproducible in vitro rapid multiplication protocol for Ethiopian black pepper species from seed would increase the social, environmental and economic benefit derived from the species. Therefore, this work was undertaken with the following objective to asses' effects of different types and concentrations of cytokinins on shoot induction and multiplication.

It was concluded that different concentration of cytokinins (kind and concentration) influenced the in vitro propagation of black pepper (Teppi local cultivar [Tato/380]) both in shoot induction and multiplication and rooting. There was a significant difference in the average number of regenerated shoots. The use of TDZ resulted in significantly lower shoot regeneration as compared to BA. Increasing the concentration of BA increased the number of regenerated shoots but decreased the number of days to shoot induction. The application of BA in the medium as compared to TDZ stimulates the rate of shoot regeneration and the greatest shoot regeneration was found on media with 4.0 mg/l of BA alone. Since BA has better effect on shoot regeneration, it is suggested that higher concentration of this growth regulator could be used to obtain the desirable shoot regeneration on black pepper.

4.2. Recommendations

It can be recommend that the in vitro propagation of black pepper using cytokinins, that is the combination of TDZ and BA for shoot induction and multiplication was not effective, except for mean number of days to shoot induction and mean number of shoot per explants. Addition of TDZ, alone was found to be effective for excess shoot regeneration. High level of BA was mostly effective on mean number of leaves per shoot and mean shoot length. The combined effect of TDZ and BA is effective for mean number of days to shoot induction and mean number of shoot per explants.

Applications of cytokinins mainly BA at 4.0 mg/l is effective for maximum shoot proliferation and multiplication.

Explants other than seed explants could be used for further development of the protocol. In addition, evaluation of cytokinins in combination with other type of hormone (cytokinins and/or Auxins) should be carried out for better result on shoot induction and multiplication.

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