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TISSUE CULTURE OF BLACK PEPPER (*PIPER NIGRUM* L.) IN PAKISTAN

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Abstract

Black pepper (*Piper nigrum* L.) the "King of Spices" is a universal table condiment. It is extensively used in Pakistani cuisines and herbal medicines and imported in bulk from neighboring countries. The black pepper vine is generally cultivated by seed because other vegetative propagation methods are slow and time consuming. Therefore the tissue culture technique is considered more efficient and reliable method for rapid and mass propagation of this economically important plant. The present study was initiated to develop protocol for micro-propagation of black pepper vine. The stem, leaf and shoot tip explants from mature vine were cultured on MS medium supplemented with different concentrations of plant growth regulators (2,4-D, BA, IBA). Best callus was produced on MS medium with 1.5 mg/l BA by shoot tip explant. Shoot regeneration was excellent on MS medium with 0.5 mg/l BA. The plantlets formed were rooted best on 1.5 mg/l IBA. The rooted plants were transplanted in soil medium and acclimatized in growth room. The plants raised were test planted under the local conditions of Hattar.

Abbreviations: MS medium: Murashige & Skooge Medium, 2,4-D: 2,4-diphenylphenoxyacetic acid, BA: 6-benzylaminopurine, IBA: Indole-3-butyric acid, IAA: Indole acetic acid, Con.: Concentration, C.V.: Coefficient of Variance, S.D.: Standard Deviation.

Introduction

Black pepper (*Piper nigrum* L.) belongs to the family Piperaceae. It is a perennial woody climbing liana. 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' (Srinivasan, 2007; Mathew *et al*., 2001). The spicy taste is mainly due to the presence of a compound Piperine. Piperine is a pungent alkaloid (Tripathi *et al*., 1996) that enhances the bioavailability of various structurally and therapeutically diverse drugs. (Khajuria *et al.,* 2002). It also contains small amounts of safrol, pinene, sabinene, limonene, caryophyllene and linaonol compound.

Plant tissue culture refers to the *In vitro* culture of plants from plant parts (tissues, organs, embryos, single cells, protoplasts, etc.) on nutrient media under aseptic conditions (Altman, 2000). *In vitro* cultures are now being used as tools for the study of various basic problems in plant sciences. It is now possible to propagate all plants of economic importance in large numbers by tissue culture.

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Black pepper is also an important traditional medicine and used to treat asthma, chronic indigestion, colon toxins, obesity, sinus, congestion, fever (Ravindran, 2000), intermittent fever, cold extremities, colic, gastric ailments and diarrhoea (Ao *et al*., 1998). It has been shown to have antimicrobial activity (Dorman & Deans, 2000). Both aqueous and ethanolic extracts of black pepper have been screened for antibacterial activity against *Bacillus cereus* and *Bacillus subtilus* (Singh *et al*., 2005; Ghori & Ahmad, 2009). Aqueous decoction of black pepper exhibited maximum effect against *Staphylococcus aureus* and found to be most active antibacterial agent against all bacterial isolates except *Salmonella typhi*, *Streptococcus intermedius*, *Streptococcus mitis*, *Streptococcus salivarius*, *Streptococcus sanguis* and *Streptococcus uberis*. (Nazia Masood *et al*., 2006).

Black pepper can be propagated by seeds, cuttings, layering, and grafting. Seed propagation often results in genetic variation due to formation of recombinants while other methods of black pepper propagation are slow and time consuming (Atal & Banga, 1962). So, there is a need to introduce efficient methods for faster propagation of black pepper. In this context, plant tissue culture is the most efficient and reliable method for rapid and mass scale production of disease free, genetically stable and identical progeny (Hu & Wang, 1983) of black pepper through out the year. Tissue culture techniques have taken significant part in clonal propagation, conservation of germplasm and plant improvement in black-pepper (Bhat *et al*., 1995; Sajc *et al*., 2000). That is why *In vitro* propagation is a superior option and alternate for traditional propagation (Abbasi *et al*., 2007; George & Sherrington, 1984).

The main objective of the present study was to prepare protocols for micropropagation of disease free plants of black pepper for introduction in Pakistan and to ensure the year round availability of identical, disease-free and high quality planting material.

Materials and Methods

The mother plants of black pepper were procured from India and the explants used for the present investigation were leaves, nodes and apical meristem of healthy black pepper plant grown in green house at Qarshi Herb Research Centre, Hattar, district Haripur. Pre-sterilized explant cuttings (6-7mm) were taken and cut into segments of 4 - 5mm each in length. Explants harvested were first washed with running tap water and then treated with household detergent for five minutes. This was followed by second washing with tap water to remove all traces of detergent. The explants were then treated with 0.1% mercuric chloride for one minute. To remove all the traces of mercuric chloride, the explants were washed three times with sterilized distilled water. Each explant was inoculated to MS medium with different concentrations of hormones. Care was taken not to dip explants completely in the medium and also tip of forceps to touch the agar medium to ensure proper nutrient supply to the explants. The cultured tubes were sealed immediately after inoculation. The same procedure was repeated for multiple shoot formation. Forceps, scalpels, needles and other instruments were disinfected by dipping in ethanol and flamed before use. All culture were grown under 16 hours light and 8 hours dark period in air conditioned growth room, illuminated by 40W (watts) white fluorescent lights. The intensity of light was regulated between 1000-2000 lux. The temperature of culture room was maintained at 25±2ºC. For shoot formation from apical meristems, different concentrations of BA (0.5, 1, 1.5, 2, 2.5 and 3 mg/l) alone were tested. Sub-culturing was carried out at every 21 days interval. Nodal segments from the

proliferated shoots were sub-cultured again for further multiple shoot induction. Regenerated multiple shoots were cut and individual shoots were placed in MS medium containing different concentrations of IBA and IAA for root induction. Data was recorded after 5 weeks for multiple shoot induction and rooting frequency. The experiment was laid out in replicated form with 30 replications. The explants which showed positive response were included in the statistical analysis for determination of standard deviation and co-efficient of variance.

Results and Discussion

In the present investigation, various growth regulators (Auxions and Cytokinins) were tested for callogenesis of black pepper vine from leaf, stem & shoot tip explants and organogenesis from callus to produce disease free plants of *Piper nigrum* L. Many successful *In vitro* techniques for micropropagation of black-pepper have already been reported using shoot-tip explant (Nazeem *et al*., 1992; Philip *et al*., 1992; Babu *et al*., 1993; Joseph *et al*., 1996; Philip *et al*., 1992), leaf explant (Sujatha *et al*., 2003), nodes explant (Bhat *et al*., 1992) root explants (Bhat *et al*., 1995) and seeds (Nair & Gupta, 2006). The plantlets regenerated from seedling derived callus and shoot tips had also been reported, but most attempts to regenerate plants from mature vine were unsuccessful (Mathews & Rao, 1984). The results of the present study clearly demonstrate the regeneration of plantlets from mature black pepper vine through tissue culture. The effect of various hormones on callogenesis and organogenesis are separately described as follows:

Effect of different concentrations of 2,4-D, on callogenesis: For *In vitro* callus formation from leaf, stem and shoot tip explants were taken from mature vine of black pepper. Murashige & Skoog Medium (1962) was supplemented with different concentrations of 2,4-D, ranging from 0.5 mg/l to 3.0 mg/l. The callus formation from leaf explant was poor (+) on concentrations 0.5, 1.0 and 1.5 mg/l of 2,4-D. while the higher concentrations of 2.0, 2.5, and 3.0 mg/l showed fair (++) callogenic response. The stem explant gave poor callus formation (+) on 1.0 and 3.0 mg/l concentrations, while it was fair (++) on rest of the concentrations of 2,4-D i.e., 0.5, 1.5, 2.0 and 2.5 mg/l. The shoot tip explant gave small amount of callus with 1.0, 2.0 and 3.0 mg/l of 2, 4-D. whereas 0.5, 1.5 and 2.5mg/l showed fair callogenic response (++). However, 2,4-D did not produce appropriate amount of callus on almost all of the concentrations and explants tested. The callus formed was hard, nonfriable, brownish white and nonembryogenic (Tables & Figs. 1, 2 & 3).

Effect of different concentrations of BA on callogenesis: The leaf explant with 0.5 to 2.0 mg/l concentrations gave fair mass (++) while 2.5 and 3.0 mg/l were poor (+) in callus formation. The results are in line with Philip (1992) who obtained calli by culturing leaf explants on MS medium supplemented with BA 1 μM along with IAA.

Stem explant showed fair response (++) at 0.5 mg/l, but 1.0 and 1.5 mg/l of BA made good callus (+++). On higher concentrations of 2.0 and 2.5 mg/l produced poor callus (+) and 3.0 mg/l yielded no callus (-). Our results are similar to Bhat (1995), who reported that at higher concentrations of BA (5-10 μM), the Callogenic response of the nodal explants of *P. nigrum* responded less.

Table 1. Callogenic response from leaf explants of *Piper nigrum* L., at different hormonal combinations / concentrations of 'Plant Growth Regulators' (PGR).

Culture number	PGR	Conc. (mg/l)	Callus formation % Avg ± SD	Callus formation C.V. %	Callogenic response*
01	2,4-D	0.5	40.00 ± 0.50	124.57	+
02	2,4-D	1.0	26.67 ± 0.45	168.67	+
03	2,4-D	1.5	16.67 ± 0.38	227.43	+
04	2,4-D	2.0	56.67 ± 0.50	88.94	++
05	2,4-D	2.5	66.67 ± 0.48	71.92	++
06	2,4-D	3.0	53.33 ± 0.51	95.14	++
07	BA	0.5	70.00 ± 0.47	66.58	++
08	BA	1.0	60.00 ± 0.50	83.05	++
09	BA	1.5	50.00 ± 0.51	101.71	++
10	BA	2.0	53.33 ± 0.51	95.14	++
11	BA	2.5	6.67 ± 0.25	380.56	+
12	BA	3.0	16.67 ± 0.38	227.43	+
13	IBA	0.5	10.00 ± 0.31	305.13	+
14	IBA	1.0	53.33 ± 0.51	95.14	++
15	IBA	1.5	60.00 ± 0.50	83.05	++
16	IBA	2.0	56.67 ± 0.50	88.94	++
17	IBA	2.5	33.33 ± 0.48	143.84	+
18	IBA	3.0	26.67 ± 0.45	168.67	+

* +: Poor, ++: Fair, +++: Good, ++++: Excellent, -: No response,

Table 2. Callogenic response from stem explants of *Piper nigrum* L., at different hormonal combinations / concentrations of 'Plant Growth Regulators' (PGR).

Culture number	PGR	Conc. (mg/l)	Callus formation % Avg ± SD	Callus formation C.V. %	Callogenic response*
01	2,4-D	0.5	63.33 ± 0.49	77.39	++
02	2,4-D	1.0	66.67 ± 0.48	71.92	+
03	2,4-D	1.5	73.33 ± 0.45	61.33	++
04	2,4-D	2.0	83.33 ± 0.38	45.49	++
05	2,4-D	2.5	60.00 ± 0.50	83.05	++
06	2,4-D	3.0	33.33 ± 0.48	143.84	+
07	BA	0.5	50.00 ± 0.51	101.71	++
08	BA	1.0	86.67 ± 0.35	39.89	+++
09	BA	1.5	96.67 ± 0.18	18.89	+++
10	BA	2.0	50.00 ± 0.51	101.71	+
11	BA	2.5	53.33 ± 0.51	95.14	+
12	BA	3.0	0.00 ± 0.00	-	-
13	IBA	0.5	20.00 ± 0.41	203.42	+
14	IBA	1.0	60.00 ± 0.50	83.05	++
15	IBA	1.5	63.33 ± 0.49	77.39	++
16	IBA	2.0	50.00 ± 0.51	101.71	++
17	IBA	2.5	16.67 ± 0.38	227.43	+
18	IBA	3.0	26.67 ± 0.45	168.67	+

* +: Poor, ++: Fair, +++: Good, ++++: Excellent, -: No response,

Table 3. Callogenic response from shoot tip explants of *Piper nigrum* L., at different hormonal combinations / concentrations of 'Plant Growth Regulators' (PGR).

Culture number	PGR	Conc. (mg/l)	Callus formation % Avg ± SD	Callus formation C.V. %	Callogenic response*
01	2,4-D	0.5	70.00 ± 0.47	66.58	++
02	2,4-D	1.0	33.33 ± 0.48	143.84	+
03	2,4-D	1.5	76.67 ± 0.43	56.11	++
04	2,4-D	2.0	36.67 ± 0.49	133.67	+
05	2,4-D	2.5	66.67 ± 0.48	71.92	++
06	2,4-D	3.0	16.67 ± 0.38	227.43	+
07	BA	0.5	83.33 ± 0.38	45.49	++
08	BA	1.0	96.67 ± 0.18	18.89	++
09	BA	1.5	100.00 ± 0.00	0.00	++++
10	BA	2.0	66.67 ± 0.48	71.92	++
11	BA	2.5	50.00 ± 0.51	101.71	+
12	BA	3.0	56.67 ± 0.50	88.94	+
13	IBA	0.5	53.33 ± 0.51	95.14	+
14	IBA	1.0	66.67 ± 0.48	71.92	++
15	IBA	1.5	70.00 ± 0.47	66.58	++
16	IBA	2.0	76.67 ± 0.43	56.11	++
17	IBA	2.5	66.67 ± 0.48	71.92	++
18	IBA	3.0	50.00 ± 0.51	101.71	+

* +: Poor, ++: Fair, +++: Good, ++++: Excellent, -: No response,

Table 4. Shoot initiation from callus explants of *Piper nigrum* L., at different hormonal combinations / concentrations of 'Plant Growth Regulators' (PGR).

Culture number	PGR	Conc. (mg/l)	Shoot regeneration % Avg ± SD	Shoot regeneration C.V. %	Shoot regeneration response*
01	2,4-D	0.5	0.00 ± 0.00	-	-
02	2,4-D	1.0	0.00 ± 0.00	-	-
03	2,4-D	1.5	0.00 ± 0.00	-	-
04	2,4-D	2.0	0.00 ± 0.00	-	-
05	2,4-D	2.5	0.00 ± 0.00	-	-
06	2,4-D	3.0	0.00 ± 0.00	-	-
07	BA	0.5	100.00 ± 0.00	0.00	++++
08	BA	1.0	96.67 ± 0.18	18.89	+++
09	BA	1.5	86.67 ± 0.35	39.89	+++
10	BA	2.0	80.00 ± 0.41	50.85	++
11	BA	2.5	50.00 ± 0.51	101.71	+
12	BA	3.0	33.33 ± 0.48	143.84	+
13	IBA	0.5	0.00 ± 0.00	-	-
14	IBA	1.0	0.00 ± 0.00	-	-
15	IBA	1.5	0.00 ± 0.00	-	-
16	IBA	2.0	0.00 ± 0.00	-	-
17	IBA	2.5	0.00 ± 0.00	-	-
18	IBA	3.0	0.00 ± 0.00	-	-

* +: Poor, ++: Fair, +++: Good, ++++: Excellent, -: No response

Table 5. Root initiation from callus explants at different hormonal combinations / concentrations of 'Plant Growth Regulators' (PGR).

Culture number	PGR	Conc. (mg/l)	Root regeneration % Avg ± SD	Root regeneration C.V. %	Root regeneration response*
01	2,4-D	0.5	66.67 ± 0.48	71.92	++
02	2,4-D	1.0	73.33 ± 0.45	61.33	++
03	2,4-D	1.5	83.33 ± 0.38	45.49	++
04	2,4-D	2.0	60.00 ± 0.50	83.05	+
05	2,4-D	2.5	40.00 ± 0.50	124.57	+
06	2,4-D	3.0	36.67 ± 0.49	133.67	+
07	BA	0.5	0.00 ± 0.00	-	-
08	BA	1.0	0.00 ± 0.00	-	-
09	BA	1.5	0.00 ± 0.00	-	-
10	BA	2.0	0.00 ± 0.00	-	-
11	BA	2.5	0.00 ± 0.00	-	-
12	BA	3.0	0.00 ± 0.00	-	-
13	IBA	0.5	36.67 ± 0.49	133.67	+
14	IBA	1.0	83.33 ± 0.38	45.49	+
15	IBA	1.5	100.00 ± 0.00	0.00	++++
16	IBA	2.0	83.33 ± 0.38	45.49	+++
17	IBA	2.5	90.00 ± 0.31	33.90	+++
18	IBA	3.0	66.67 ± 0.48	71.92	+

* +: Poor, ++: Fair, +++: Good, ++++: Excellent, -: No response

Almost similar results were obtained with shoot tip explant, where 0.5, 1.0 and 2.0 mg/l made fair amount of calli (++) and again 3.0 mg/l showed no response (-). Our results are in agreement with Philip (1992) who reported that BA alone or in combination with Indole butyric acid (IBA) and Adenosine sulphate (AdSO4) supports initial proliferation of shoot tip explants. More than 5 µM of BA suppresses growth of shoots and proliferation. Excellent result (++++) was obtained with 1.5 mg/l of BA in shoot tip and 100% Callogenic response with embryogenic cells was achieved (Tables & Figs.1, 2 & 3). Similar results have been reported by Philip (1992) with 1.5 µM BA and 3 µM IBA. All the calli obtained were of brownish green color, friable and embryogenic.

Effect of different concentrations of IBA on callogenesis: The callus formation by leaf explant on 0.5, 2.5 and 3.0 mg/l of IBA was poor (+) and 1.0, 1.5 and 2.0 mg /l made fair mass (++) of callus. Fair Callogenic response (++) was observed at 1.0, 1.5 and 2.0 mg/l of IBA as compared to 0.5, 2.5 and 3.0 mg/l, which showed poor callus (+) formation with stem explants. The shoot tip explants made poor (+) calluses on 0.5 and 3.0 mg/l while fair (++) amount of callus was produced on rest of the concentrations of IBA i.e., 1.0, 1.5, 2.0, and 2.5 mg/l. The overall results indicated that IBA did not perform well for callus production on all of the hormonal concentrations and explants tested. The callus formed was hard, brownish yellow and not embryogenic (Tables & Figs. 1, 2 & 3).

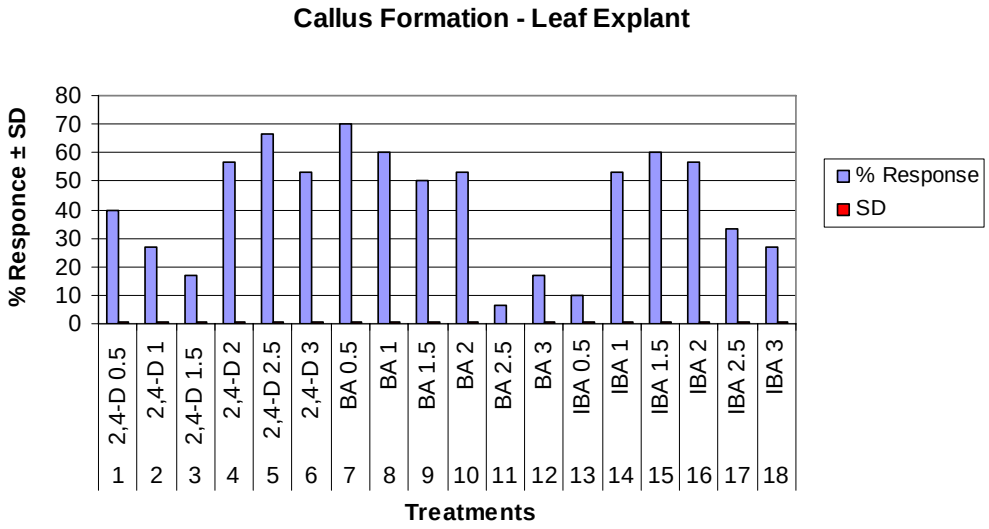

Fig. 1. Callogenic response from leaf explants of *Piper nigrum* L., at different hormonal combinations / concentrations of 'Plant Growth Regulators' (PGR).

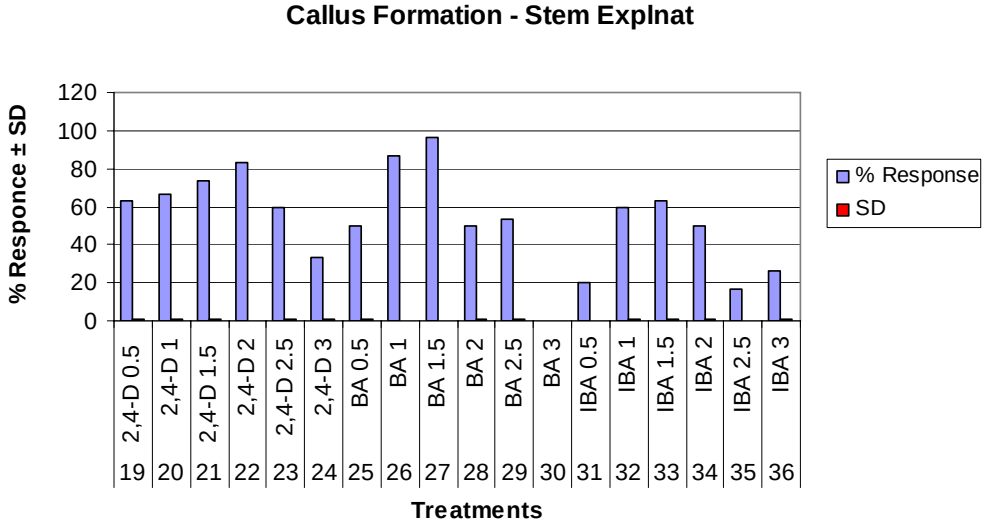

Fig. 2. Callogenic response from stem explants of *Piper nigrum* L., at different hormonal combinations / concentrations of 'Plant Growth Regulators' (PGR).

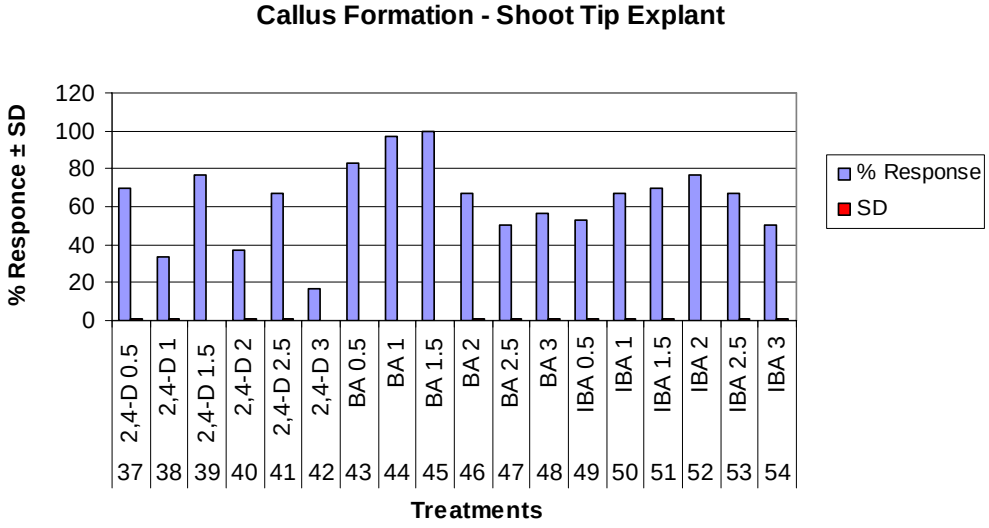

Fig. 3. Callogenic response from shoot tip explants of *Piper nigrum* L., at different hormonal combinations / concentrations of 'Plant Growth Regulators' (PGR).

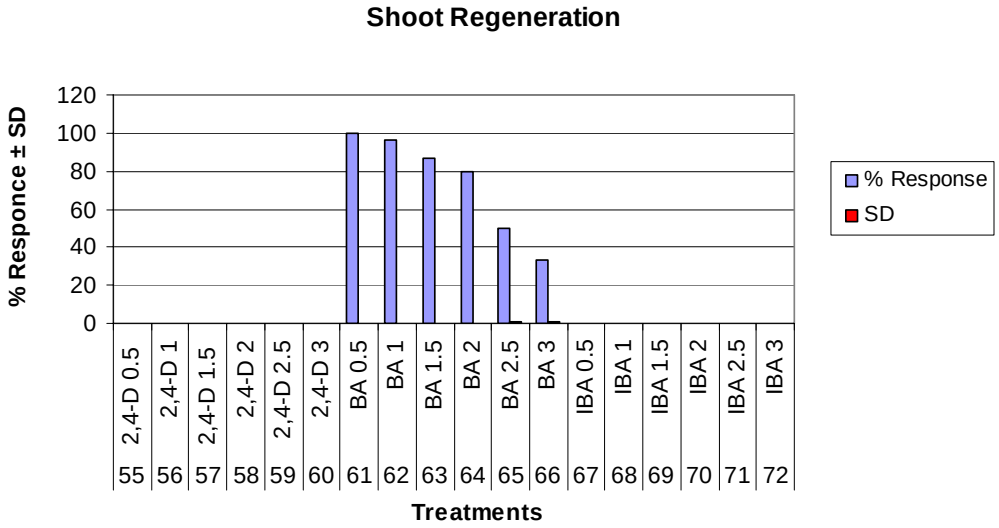

Fig. 4. Shoot regeneration from callus explants of *Piper nigrum* L., at different hormonal combinations / concentrations of 'Plant Growth Regulators' (PGR).

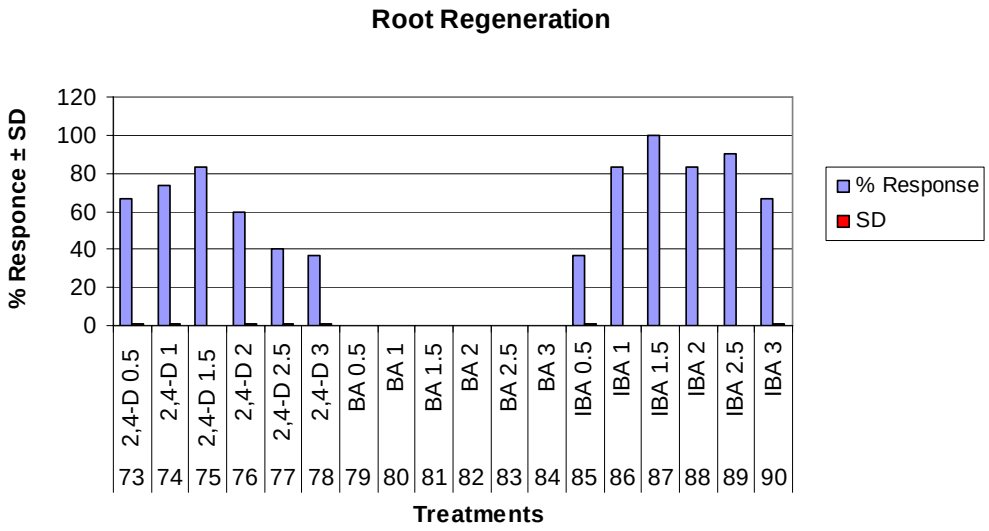

Fig. 5. Root initiation from callus explants at different hormonal combinations / concentrations of 'Plant Growth Regulators' (PGR).

Effect of different concentrations of 2,4-D, BA and IBA on organogenesis: With Different concentrations of auxins i.e., 2,4-D and IBA (ranging from 0.5–3.0 mg/l) on MS medium, no shoot regeneration response (-) was seen from callus explant. The results are in agreement with Anand & Rao (2000) who observed that the shoot tip and nodal explants failed to stimulate shoot regeneration on cytokinin-free medium. The effect of different concentrations of BA on shoot formation from callus is presented in Table 4. The results show that when 0.5 mg/l of BA was used in MS medium, 100% shoot formation (++++) was obtained after 14 days of callus inoculation. Good (+++) shoot formation was noticed at 1.0 & 1.5 mg/l and fair (++) with 2.0 mg/l of BA. The results are in contradiction to Rubluo and Barroso (1992) who reported BA and IAA considerably ineffective in shoot formation. At higher concentration, the rate of organogenesis decreased gradually. The shoot initiation was poor with 2.5 and 3.0 mg/l BA which indicate that higher the concentration of BA lowers the rate of organogenesis. Anand & Rao (2000) also reported that increasing the concentration of BA from 0 to 6.6

µM enhanced regeneration but further increase in the concentrations of BA and kinetin suppressed number of shoot formation.

The different concentrations of BA (cytokinin) ranging from 0.5–3.0 mg/l on MS medium produced no roots (-) from the regenerated shoots because the process of *In vitro* root initiation, development and elongation normally require medium containing Auxin (Azad *et al*., 2003).

When lower concentrations of the 2,4-D (0.5, 1.0 and 1.5 mg/l) were used, fair amount (+ +) of roots were formed and the increase in concentrations from 2.0, 2.5 to 3.0 mg/l, root initiation was poor (+). Lower concentrations of IBA initiated poor (+) roots in most of the cultures. The concentrations of 2.0 and 2.5 mg/l produced good (+++) roots, whereas the higher concentration of 3.0 mg/l formed poor roots (+). Kunisaki *et al*., (2003) reported that the micro shoots obtained by *In vitro* shoot regeneration were rooted in ½MS basal medium in case of *Piper methysticum.*

Excellent rooting (++++) was shown by 1.5 mg/l IBA on MS medium in all cultures within 8 days (Table & Fig. 5). Contrary to our results, Bhat (1995); Azad (2003) used 1 µM IAA for root production from the regenerated shoots.

Rooted plantlets were removed from the medium and transferred to pots containing compost, sand, and soil media in 1:1:1 ratio. The plants were covered with transparent polythene bags for two weeks for acclimatization (Philip *et al*., 1992). Anand & Rao (2000) reported the use of growth chamber under high humidity conditions for acclimatization of black pepper plants. Polythene covers were gradually removed over a period of 2 weeks (Bhat *et al*., 1995). The tissue cultured plants of black pepper were acclimatized according to the climatic conditions of Hattar Industrial Estate, KPK, Pakistan, especially for atmospheric humidity and temperature.

The rooted plants were transferred to green house for four weeks and then successfully test planted in the fields at Hattar. Black pepper being a vine needs support for climbing. Four different tree species were selected to provide support for vines i.e., *Eucalyptus camaldulensis, Grevillea robusta, Pinus roxburghii* and *Phyllanthus emblica.* The vine grew best on Silver Oak (*Grevillea robusta*) tree round the year. Black pepper vine is frost tender. The vines growing in green house during winter season had better health than those growing open in air.

Conclusion

The present study can be used as a protocol for the tissue culture of black pepper *In vitro* conditions *via* callus and meristem culture. Tissue cultured plants should be acclimatized before introduction to field and must be planted in the fields with minimum frost areas or else inside green house.

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