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Effect of Explants and Genotypes on Primary Somatic Embryogenesis in Black Pepper (*Piper nigrum* L.)

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Summary Influence of various fruit-derived explants and different genotypes on induction of somatic embryogenesis in black pepper was studied. Among the various explants cultured on plant growth regulator-free solid SH medium maintained in dark, the 'abortively germinated seeds *in vitro*' produced the highest percent response as well as number of somatic embryos per responded explant. Lowest percent response and frequency of somatic embryos were evident with the 'unripened green fruits with zygotic embryo removed'. Zygotic embryos as such failed to produce any somatic embryogenic response. Of the various genotypes tested, cultivar 'Karimunda' was found to be highly embryogenic and cultivar 'Kutching' was totally non embryogenic. Other cultivars showed variable degree of embryogenic response.

Key words Black pepper, Explants, Genotypes, *Piper nigrum*, Somatic embryogenesis.

Black pepper (*Piper nigrum* L., Piperaceae) has a prominent place among the spices grown in India and is widely used in culinary preparations, food processing, perfumery, and also as a condiment throughout the world. India is a major producer and exporter of black pepper, the annual export being over Rs. 4000 million. In spite of its great economic importance, the productivity of black pepper in India is declining presently, mainly because of non-availability of adequate planting material of high yielding varieties and crop losses due to diseases, nematodes and insect pests. Vegetative propagation through conventional techniques is inadequate to meet the increase in demand for planting material. Propagation through seed is not advisable as it yields only heterogeneous progenies (Ravindran *et al.* 2000). An efficient, high frequency *in vitro* plant regeneration system, preferably from single cells is essential for the application of modern methods of gene transfer using *Agrobacterium* based vectors or through micro-projectile bombardment. Such a system may also be helpful for large scale micropropagtion.

In vitro plant regeneration of black pepper through shoot organogenesis (Mathews and Rao 1984, Philip *et al.* 1992, Bhat *et al.* 1995) as well as somatic embryogenesis (Joseph *et al.* 1996, Nair and Dutta Gupta 2003) has been reported. Micropropagation through somatic embryogenesis is usually preferred over organogenesis, as more regenerated plants can be obtained from few or single cells which increases the scope for getting transformed plants in gene transfer experiments. Direct somatic embryogenesis from vegetative and seed tissues other than zygotic embryos and endosperm can be utilized as a method for large-scale clonal propagation system for multiplying elite genotypes and high yielding varieties.

Direct somatic embryogenesis from the sporophytic tissues of germinating seeds of black pepper (*Piper nigrum*) and their ontogeny from single cells have been reported by Nair and Dutta Gupta (2003). A report is also available on callus mediated somatic embryogenesis from zygotic

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embryos (Joseph *et al.* 1996). The capacity for somatic embryogenesis is influenced by several factors, of which explant type and genotype are very important (George 1993, Litz and Gray 1995, Arnold *et al.* 2002). A proper understanding of the influence of these factors in a particular plant species will help to scale up the process of somatic embryogenesis resulting in increased frequency of plant regeneration. Effect of explants on somatic embryogenesis has been recorded in several plants such as *Capsicum annum* (Park *et al.* 1992), *Gossypium hirsutum* (Trolinder and Goodin 1988), *Gentiana* spp. (Mikula and Rybczynski 2001), *Pisum sativum* (Doorne *et al.* 1995) and *Solanum melongena* (Sharma *et al.* 1995). Influence of genotypes on somatic embryogenesis is also well documented (Parrott *et al.* 1989, Trolinder and Xhixian 1989, Ozias-Akins *et al.* 1992, Bailey *et al.* 1993, McKentley 1995, Sharma *et al.* 1995, Kikkert *et al.* 1997, Kintzios and Taravira 1997, Litz *et al.* 1998, Meurer *et al.* 2001, Tomlin *et al.* 2002). Though a few reports on the effect of explants and genotypes on shoot organogenesis are available in black pepper (Bhat *et al.* 1995, Rajasekharan and Kumar 1997), such information on somatic embryogenesis was not available so far.

In continuation to the previous work on direct somatic embryogenesis from tissues of germinating seeds (Nair and Dutta Gupta 2003), the present study focused on testing the suitability of different fruit-derived explant types and influence of different genotypes on induction of somatic embryogenesis in black pepper.

Materials and methods

Plant materials

Black pepper fruits/seeds used to initiate embryogenic cultures were derived from different cultivars grown in the germplasm repository at Indian Institute of Spices Research, Calicut. Explants from the cultivar 'Karimunda' were used to test the effect of different explant types on somatic embryogenesis and 15 cultivars including 'Karimunda' were tested for the effect of genotypes on somatic embryogenesis using the potential seed explants.

Establishment of embryogenic cultures

For induction of somatic embryogenesis the method standardized by Nair and Dutta Gupta (2003) was used with appropriate modifications. The same medium and culture conditions were used for *in vitro* germination of seeds as well as for inducing somatic embryos. Seeds/fruits collected from field grown plants were washed under tap water and surface sterilized with 0.1% mercuric chloride for 10 min, followed by repeated washings (3–4 times) with sterile double distilled water prior to *in vitro* germination or direct use as explants. The seeds from *in vitro* germination cultures were used directly as explants without further sterilization.

The explants were cultured on full-strength SH (Schenk and Hildebrandt 1972) medium containing 0.8% (w/v) agar (Bacteriological grade, Hi-media) and sucrose 30 g/l without any plant growth regulators. The pH of the medium was adjusted to 5.9 prior to autoclaving. Cultures were maintained under 24 h dark period at 25±1°C. Culture tubes (25×150 mm) containing 15 ml medium was used for testing the effect of explants and Erlenmeyer flasks (250 ml) containing 50 ml medium was used for testing the genotypes. Induced somatic embryos were regenerated into plantlets in liquid SH medium (suspension) under diffused light as described by Nair and Dutta Gupta (2003).

Effect of explants on somatic embryogenesis

To select the best explant for somatic embryogenesis, an experiment was conducted with various explants such as freshly harvested intact seeds (**A**), fresh *in vitro* germinated seeds with folded cotyledons and attached seed coat (**B**), abortively germinated seeds *in vitro* (**C**), unripened green

fruits with zygotic embryo removed (**D**) and zygotic embryos scooped out from the fresh ripened seeds (**E**). The black pepper fruit is composed of a single seed derived from orthotropic ovule and the only difference between fruit and the seed is a thin fruit wall covering the seed. The fruit wall covering unripened seed is green in colour and it will turn red/orange on ripening. To extract seeds, the ripened fruits will be soaked in water and rubbed between hands to remove fruit wall.

All explants were derived form the most popular cultivar 'Karimunda'. Chalazal region of seeds in **A**–**D** were slightly cut before inoculation, for better penetration of the medium. Fruit wall at the micropylar end of green fruits in **D** were cut so as to expose micropylar tissues and zygotic embryos were removed before inoculation. Zygotic embryo (**E**) as such was included as an explant to confirm the existing report of callus mediated somatic embryogenesis (Joseph *et al.* 1996). The cultures were regularly observed for somatic embryogenesis and transferred to fresh medium of the same composition, at an interval of 30 d. Percentage of explants with somatic embryos and number of somatic embryos in each responding explant were recorded after 90 d of culture. There were 10 replicates per treatment with one explant each and the experiment was repeated five times. Somatic embryogenesis from different types of seed explants were observed and photographed under appropriate magnifications using Nikon-SMZU stereo-zoom microscope.

Influence of genotypes on somatic embryogenesis

Seed explants derived from germination cultures of black pepper cultivars such as Jeerakamundi, Kalluvally, Karimunda, Kutching, Kuthiravally, Narayakodi, Neelamundi, Neyyattinkaramunda, Panniyur-1, Perambramunda, Sreekara, Subhakara, Thevanmundi, Thommenkodi and Vadakkan were cultured for somatic embryogenesis following the method described previously (Nair and Dutta Gupta 2003). There were three replicates containing 30 seeds each per genotype. The experiment was repeated 5 times. Data on frequency of embryo induction and number of embryos per explant were recorded after 90 d of culture.

Statistical analysis

Mean and standard error were calculated for frequency of embryo induction as well as number of embryos per explant in both the experiments. The data collected were subjected to analysis of variance (ANOVA) using MSTATC software package. Data on percentages were subjected to angular transformation before analysis. Means were separated using Duncan's Multiple Range Test at $P=0.05$.

Results

Effect of explants on somatic embryogenesis

Different explant types responded variably for induction of primary somatic embryos on plant growth regulator-free SH medium. Somatic embryo initiation in **A**-type explant was started by 60 d of culture, after germination of most of the seeds. In **B** and **C**-type explants somatic embryo initiation was evident by 30 d of culture. The **D**-type explant showed somatic embryo initiation only after prolonged culture period of 80 d. Zygotic embryo explant (**E**) failed to produce any somatic embryogenic response till the completion of the experiment.

Somatic embryo initiation was observed from swollen micropylar tissues of seed in all the responded explant types (Fig. 1a–e). In **A** and **B** type explants somatic embryos emerged randomly from the outer region of micropylar tissue in the same pattern as described by Nair and Dutta Gupta (2003) (Fig. 1b). The seed explants showing various degrees of abortive germination (**C**) produced somatic embryos either all around the swollen micropylar tissue (Fig. 1c), randomly on the micropylar tissue (Fig. 1d) or from the extreme tip of the micropylar tissue (Fig. 1e). The unripened

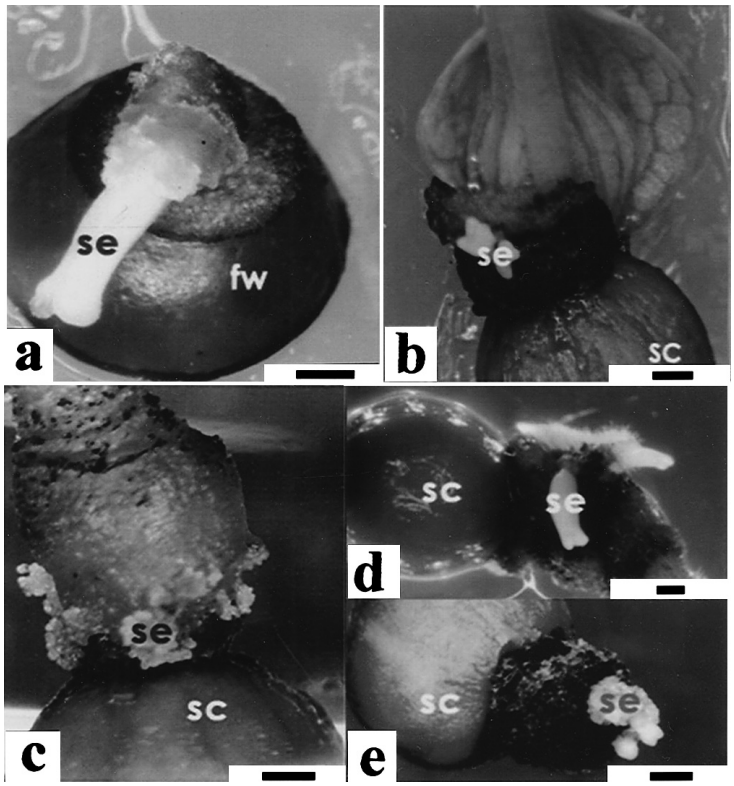

Fig. 1. Primary somatic embryogenesis from different types of fruit-derived explants in black pepper after 90 d of culture. **a**. An unripened green fruit showing somatic embryogenesis from micropylar tissue (Zygotic embryo was removed before inoculation). **b**. A normally germinated seed showing somatic embryogenesis from the micropylar ring tissue. **c**, **d** & **e**. Seeds at different stages of abortive germination showing somatic embryogenesis from micropylar ring tissue. Bars represent 1 mm. (se—Somatic embryos; fw—Fruit wall; sc—Seed coat).

Table 1. Effect of different fruit-derived explants on primary somatic embryo induction in black pepper 'Karimunda' on plant growth regulator-free SH medium after 90 d of culture

Explant type	Percent somatic embryogenesis±SE	No. of somatic embryos per responding explant±SE
A	16.0±2.449 b*	4.22±0.38 c*
B	24.0±4.000 b	6.20±0.37 b
C	32.2±2.000 a	10.20±0.66 a
D	5.0±1.581 c	2.00±0.32 d
E	0.0±0.000 d	0.00±0.00 e

* Means followed by the same letter are not significantly different at $p=0.05$ of Duncan's Multiple Range Test.

green fruits (**D**) produced somatic embryos from the exposed micropylar tissue in a very low frequency (Fig. 1a). The zygotic embryo explants (**E**) developed themselves into small less vigorous plantlets without producing any somatic embryos or embryogenic callus.

Among the explants tested, highest percent response (32.2) and number of primary somatic embryos per responded seed (10.20) were obtained with **C**-type explants followed by **B** and **A**. Lowest percent response and frequency of somatic embryos were evident with type **D** explant. The details are presented in Table 1.

Table 2. Influence of genotype on induction of somatic embryos from germinating seeds of black pepper on plant growth regulator-free SH medium after 90 d of culture

Genotype (Cultivar)	Percent somatic embryogenesis±SE	No. of somatic embryos per responding seed±SE
Jeerakamundi	11.0±1.00 f*	3.4±0.24 f*
Kalluvally	17.0±1.22 e	4.2±0.37 def
Karimunda	28.0±1.23 a	7.0±0.55 a
Kutching	0.0±0.00 g	0.0±0.00 h
Kuthiravally	23.0±1.22 bc	5.4±0.24 bc
Narayakodi	18.0±1.22 de	4.2±0.73 def
Neelamundi	19.0±1.00 cde	6.2±0.37 ab
Neyyattinkaramunda	22.0±2.00 bcd	5.8±0.20 bc
Panniyur-1	26.0±1.00 ab	3.8±0.37 f
Perambramunda	23.0±1.22 bc	3.6±0.24 f
Sreekara	23.0±1.22 bc	4.0±0.32 ef
Subhakara	25.0±2.24 ab	5.0±0.45 cde
Thevanmundi	11.0±1.00 f	3.4±0.24 f
Thommenkodi	22.0±1.22 bcd	5.2±0.27 bcd
Vadakkan	10.0±1.58 f	1.6±0.24 g

* Means followed by the same letters are not significantly different at $p=0.05$ of Duncan's Multiple Range Test.

Influence of genotypes on somatic embryogenesis

The extent of somatic embryogenesis varied extensively with the genotype (Table 2). Of the 15 genotypes tested, 14 showed the embryogenic response. Malaysian cultivar 'Kutching' has not exhibited any somatic embryogenic response. Among the responded genotypes, 'Karimunda' exhibited the highest frequency of embryogenesis (28.0%) and number of somatic embryos per responded explant (7.0). Embryogenic response varied between 22–26% in the cultivars 'Panniyur-1', 'Subhakara', 'Sreekara', 'Perambramunda', 'Kuthiravally', 'Neyyattinkaramunda' and 'Thommenkodi'. Moderate response of 11–19% was observed in 'Neelamundi', 'Narayakodi', 'Kalluvally', 'Thevanmundi' and 'Jeerakamundi'. The number of embryos per responding seed explant also varied considerably in these cultivars (Table 2). In cultivar 'Vadakkan' only 10% of the seeds responded for somatic embryogenesis and the mean number of embryos per explant also found to be very low (1.6).

Germination of somatic embryo

Somatic embryos derived from both the experiments germinated into plantlets with 95% efficiency.

Discussion

Improvement of an *in vitro* plant regeneration system demands a clear understanding on various factors influencing the process of induction, proliferation and scaling up. This is true with somatic embryogenesis also. Among the major factors influencing induction of somatic embryogenesis, the type of explant and genotype selected play a vital role in achieving successful results (George 1993, Narayanaswamy 1994, Lo schiavo 1995). Direct somatic embryogenesis from micropylar tissues of the germinating black pepper seeds on plant growth-regulator free SH medium, in the absence of light was described by Nair and Dutta Gupta (2003). The present study compares the potential of different fruit-derived explants and genotypes of black pepper in the induction of somatic embryogenesis.

A differential response for somatic embryogenesis was observed in various explants tested, in-

dicating the tissue and stage specificity. The black pepper seeds showing abortive germination *in vitro* (**C**) was found to be the best starting material for inducing primary somatic embryogenesis compared to other fruit-derived explants. This may be due to the fact that the failure of development of zygotic embryos into seedling might have stimulated more pre-embryogenic determined cells (PEDCs) in the micropylar tissue to select the alternative path of embryogenesis *in vitro*. Moreover, seed-derived nutrients and internal growth regulators, which would have been available for the growth of zygotic embryos otherwise, might have been utilized by the PEDCs to produce somatic embryos. The stage of abortive germination has not affected the potential for somatic embryogenesis in the case of **C**-type explant. Somatic embryogenesis was observed in various types of abortively germinated seeds such as 'root and part of hypocotyls only emerged' (Fig. 1c), 'root only emerged' (Fig. 1d) and 'micropylar tissue swollen up for germination but not further progressed' (Fig. 1e). The more time taken for initiation of somatic embryogenesis in the case of fresh seeds (**A**) may be due to the time required for germination compared to **B** and **C**-type explants which were in the process of germination at the time of explanting itself. Thus, it is evident that there is no additional advantage by using fresh seeds compared to seed explants derived from *in vitro* germination cultures. The unripened green fruits (**D**) produced only very few somatic embryos from a low frequency of explants. This indicates that micropylar tissues in unripened fruits are having only very few cells in a suitable physiological stage for the induction of somatic embryogenesis. This is in contradiction to the conventional belief that relatively younger tissues will give better response for somatic embryogenesis (George 1993). Buckley and Trigiano (1994) obtained somatic embryogenesis from integuments of ovules with more relative maturity than very young ovules in *Cercis canadensis*. Paraa and Amo-Marco (1998) observed that 2 months old immature seeds responded better for primary somatic embryogenesis in *Myrtus communis* compared to hypocotyls and cotyledon explants from seedlings. The zygotic embryo explants (**C**) used in the present study failed to elicit any somatic embryogenic response. Conversely, Joseph *et al.* (1996) reported callus-mediated somatic embryogenesis from zygotic embryos of mature seeds cultured on plant growth regulator-free SH medium. Reasons for such contradictory result are not clear. The tissues from different regions of the same organ can differ in their ability to produce somatic embryos. Yadav and Rajam (1997) observed that in *Solanum melongena* 'Pusa Purple Long', explants from different regions of the leaf differed significantly for embryogenic potential. They attributed this to the difference in spatial distribution of free and conjugated polyamines at different regions of the leaf. Several reports are available on the influence of explants in the induction of somatic embryogenesis in a range of plant species (Trolinder and Goodin 1988, Park *et al.* 1992, Sharma *et al.* 1995, Doorne *et al.* 1995, Holme and Petersen 1996, Mikula and Rybczynski 2001). The effect of explant on somatic embryogenesis in black pepper is, as far we know, now reported for the first time.

A significant influence of genotypic difference on induction of primary somatic embryogenesis of black pepper was evident from the present study. Of the different genotypes tested, cultivar 'Karimunda' was found to be highly embryogenic, 'Vadakkan' poorly embryogenic and Malaysian cultivar 'Kutching' non-embryogenic. Other cultivars exhibited variable embryogenic responses (Table 2). The reason for high embryogenic potential of 'Karimunda' may be the stability of this cultivar, being the most popular and ancestrally cultivated one in the black pepper growing state of Kerala, India (Ravindran *et al.* 2000). Besides, this cultivar was utilized originally to standardize the present system of direct somatic embryogenesis from tissues of germinating seeds (Nair and Dutta Gupta 2003). Non-embryogeic nature of 'Kutching' may be an indication of wide genetic divergence of this Malaysian cultivar from the Indian cultivars, which was reflected in the potential for somatic embryogenesis also. Low embryogenic potential of 'Vadakkan' may be attributed to its polyploid nature ($2n=78$) compared to the other diploid cultivars ($2n=52$) stiudied. Polyploidy in this cultivar has already been reported (Nair *et al.* 1993).

The ability of closely related plants to produce somatic embryos directly on explants is under

genetic control and differences between varieties are often found (Parrott *et al.* 1991, George 1993, Narayanaswamy 1994). For example, two dominant genes are necessary for an intermediate frequency of somatic embryogenesis, while the presence of a third dominant gene provides a high frequency of regeneration in cucumber (Parrott *et al.* 1991). Stamp and Meredith (1988) obtained somatic embryos on the zygotic embryos of four cultivars of *Vitis vinifera*, but could not induce them to form on cultivar 'Pinot Noir'. The direct formation of somatic embryos on apple leaf segments was genotype-dependent (Welander 1988). Such genotype-specific variation of somatic embryogenesis has also been reported in other crop plants like cotton (Trolinder and Xhixian 1989, Kumar *et al.* 1998), potato (Seabrook and Douglass 2001), peanut (Chengalrayan *et al.* 1998) and soybean (Parrott *et al.* 1989, Bailey *et al.* 1993, Li and Grabau 1996). Even though a few reports on the influence of genotypes on shoot proliferation in black pepper is available (Joseph 1995, Rajasekharan and Kumar 1997), such report on somatic embryogenesis was lacking so far. Thus the present report is the first of its kind.

The information generated on the suitability of explants and influence of different genotypes on primary somatic embryogenesis of black pepper may be helpful in improving the existing protocol (Nair and Dutta Gupta 2003) for its further utilization in gene transfer experiments or commercial micropropagation.

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