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A highly efficient and improved protocol for exploitation of somaclonal variation for enhancing alien gene introgression in wide cross hybrid of *Oryza sativa/Oryza brachyantha*

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The objective of the present study was to develop an effective protocol for optimum callus induction, duration of culture and size of explants of primordial panicle of inter-genomic hybrid of Oryza *sativa*/ Oryza *brachyantha*. It was observed that, primordial panicle at very young stage (0.5 to 1.5 cm) takes hardly 5 to 7 days for callus initiation. The two basic media tested, the Murashige and Skoog (MS) medium and Chu's N_6 medium supplemented with 2-4-D, NAA and kinetin (2 mg L^{-1} 2 mg L^{-1} and 1 mg L^{-1}) was better for callus growth and proliferation. In this medium, callus was sub-cultured continuously for 12 passages (12 months) without loss of totipotency. Plantlet regeneration in MS medium was examined using two cytokinins (KIN and BA) along with one auxin NAA at various concentrations and combinations. Tests indicated that, good plant regeneration could be best effected without BA, but with 1.5 to 2.0 mg L^{-1} of KIN at fixed level of NAA (at 0.5 mg L^{-1}).

Key words: Somaclonal, callus, subculture, wide cross, MS medium, N_6 medium.

INTRODUCTION

Improvement in plant breeding techniques in present century have resulted in increased yields and solved many problems associated with disease, insects, harvest, and quality. The plant breeders have, historically, utilized the variability in land races for selection and improvement of crops. However, as modern varieties are planted on much of the cultivated acreage and as human population centres expand, many land races that were developed by our ancestors, are no longer grown and the associated wild species which coexisted with land races in their natural habitat are becoming extinct. In addition, the variability and germplasm resources available for many cultivated varieties are becoming extremely limited (Harlan, 1976). As additional genetic resources are required to enrich the germplasm, unique and imaginative procedures are required to exploit fully the potential of our crop plants. Utilization of wild species, therefore, is one method designed to introduce additional germplasm into cultivated varieties (Stalker, 1980). The reason for leaving the wild species until last decade is well enough known as listed by (Harlan, 1976) who stated that:

(a) Wild species are often more difficult to cross and the hybrids when formed may be completely or partially sterile
(b) There may be difficulties with various ploidy levels And consequent sterility
(c) A knowledge of the taxonomy, phylogeny a

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geographical distribution of the wild species is often needed before the best use of them can be made and this knowledge is often lacking
(d) Most important of all, wild species possess a whole syndrome of undesirable characters, like shattering, awns, high foliage, low yield, poor flavour and unfavorable agronomic features long with many desirable traits for biotic and abiotic stresses.

This means that, a long and tedius programme of back crossing to the cultigen is necessary with selection for the one feature of interest from the wild species at each backcross generation. Linkages pose serious handicap but it can be broken with difficutly (Harlan, 1976; Stalker, 1980). The wild relatives of cultivated rice are a rich source of genes for resistance to diseases and pests (Heinrichs et al., 1985; Jena and Khush, 1984; Khush et al., 1977, Amante et al., 1990) and many other morphological and physiological traits for high harvest index and photosynthetic efficiency (Swaminathan, 1988). However, efforts to introgress useful genes from wild species to cultivated rice have started only during last decade and therefore very few examples of alien transfer of useful genes have been reported so far.

A gene for grassy stunt virus resistance from one Assam collection of Oryza nivara has been transferred to cultivated rice (Khush et al., 1977).A cytoplasmic factor from O. perennis to develop cytosterile lines for hybrid seed production has also been reported (lin and Yuan,1980).Wide hybridization using Oryz longistaminata and Oryz rufipogon with Oryza sativa (Taillebois, 1983) have been resulted in development of cytoplasmic male sterile lines for use in hybrid rice production. Still inumerable alien traits of interest remain untapped in Oryza. In a recent compilation of useful Oryza germplasm, (Vaughan (1989) rightly stated that, “the chance of finding resistance to insect pests of rice is about 50 times greater in wild Oryza species than the cultigen”. The genus Oryza has about 20 species of which two are cultivated. O. sativa, the Asian cultivated rice is cultivated throughout the world and glaberrima glaberrima is limited to West Africa. They have their close wild relatives, O. nivara, O. rufipogon in Asia and Oryza barthii and O. longistaminata in Africa. In cuba, there is also one species designated as Oryza glumaepatula (formerly O. cubensis) and one Oryza meridionalis in Australia. All these share a common genome which is designated AA. They are all diploid (2n = 24) and are easily crossable and interspecific hybrid within this genome show regular chromosome pairing and recombination, although inter varietal hybrid sterility, gametic sterility genes partial desynaptic genes impose certain problems in low recovery of recombinant phenotypes. Growing large population and selecting desirable parents may circumvent these problems. Thus, gene transfer between these species can be achieved easily and priority should be given for alien gene transfer through wide hybridization as advocated by (Sitch et al., 1989).

Exploitation of somaclonal variation has been demonstrated in a number of plant species including cereals such as wheat, corn and rice (Larkin and Scowcroft, 1981; Larkin et al., 1984). The origin of somaclonal variation, though precisely not known, is postulated to have arisen due to changes in chromosome structure and number, cryptic chromosome rearrangement, changes in transposable elements, somatic reduction and gene amplification and deletion (Larkin and Scowcroft, 1981).

Somaclonal variation has been employed for enhancing bivalent formation in a sterile hybrid of *Hordeum vulgare* × *H.jubetum* Orton (1980) and Lapitan et al. (1984) reported a high degree of chromosome structural changes in amphidiploids of wheat × rye. Recovery of regenerants with 24 pair having alien chromosome insertion from Oryza *latifolia* into *O. sativa* also has been reported. This technique therefore, seems promising in enhancing frequency of genetic exchange between alien and cultivated genome in wide hybridization programme of rice.

With refinement of tissue culture technique and advent of new genetic engineering tools, incorporation of genes from diverse sources have been increasingly used in crop improvement and rice being the world’s premier crop is no exception to it and in the national and international funding agencies has come forward to sponsor many crop improvement programmes in rice. Global rice biotechnology programme of Rockefeller foundation have come out with massive support and promising results have been obtained. In all these wide hybridization is one of the key components in programme aiming at transferring alien genes from diverse sources ser mounting sexual barriers. Now wide hybridization derives support of *in vitro* and *in vivo* fertilization, embryo rescue, anther and ovule cultures, chromosome engineering including amphidiploidy and aneuploidy induction and utilization, somatic hybridization, protoplast fusion and transformation and recombinant DNA technology and somatic cell culture. This along with a well defined conventional breeding programme is going to revolutionise the rice improvement.

Keeping in view of the problems encountered in alien gene transfer through wide hybridization and the emerging scenario of the rice tissue culture and biotechnology programme integrated with breeding and genetics, the present research programme on wide hybridization in rice was undertaken with the following objectives.

MATERIALS AND METHODS

Collection of inflorescence

Healthy tillers containing immature inflorescence (between 0.5 to 2.5 cm size) of F1 wide cross hybrid of *O. sativa/O. brachyantha* were collected.

Table 1. Effect of growth regulators of callus induction frequency of immature inflorescence of *sativa* / *brachyantha* hybrid.

Growth hormones (mg L^{-1})			Callus induction frequency (%)		Peak callusing duration (days)		Proportion of embryogenic calli (%)	
2,4-D	KIN	NAA	MS	N_6	MS	N_6	MS	N_6
1.0	0.5	-	26	16	20	23	10	6
1.5	0.5	-	44	24	17	19	22	14
2.0	0.5	-	54	48	19	30	30	20
2.5	0.5	-	46	60	17	30	20	11
1.0	1.0	-	20	14	17	15	4	-
1.5	1.0	-	64	44	13	13	36	16
2.0	1.0	-	100	74	7	9	90	56*
2.5	1.0	-	76	64	10	12	34	30
1.0	-	1.0	20	14	18	13	-	-
1.5	-	1.0	24	24	15	11	14	4
2.0	-	1.0	42	42	17	19	-	16
2.5	-	1.0	36	36	13	22	24	8
1.0	-	2.0	22	10	17	25	6	-
1.5	-	2.0	42	26	19	21	6	2
2.0	-	2.0	34	38	17	19	14	4
2.5	-	2.0	30	34	22	22	20	14
1.0	-	2.0	26	22	21	15	-	-
1.5	-	2.0	56	42	13	11	20	18
2.0	-	2.0	100	88	7	8	94	66
2.5	-	2.0	76	58	9	11	42	30

*Concentration of sucrose was kept constant at 3% (W/V).

Surface sterilization and incubation

Immature inflorescence (between 0.5 to 2.5 cm size) of F1 of *O. sativa/O. brachyantha* were collected by cutting below the upper most visible node approximately at the time of swelling for booting. After stripping off their outer leaves, the collected tillers were surface sterilized by two step procedure. First, after removing outer whorls of leaves and sheaths, intact tillers were dipped in 70% (v/v) ethanol for 3 min. Portions of the tillers (10 cm above the node) containing immature inflorescence were excised. Selected segments were sterilized in commercial bleach solution Teepol, 50% (v/v) for 1 h, followed by five thorough washings in sterile distilled water to remove the bleach. Second immature inflorescences of the sizes ranging from 0.5 to 2.5 cm or more were cut, and asceptically excised and individually cultured in culture tubes of 25 × 150 mm size containing suitable medium. The analysis of the data was done using statistical software SAS 9.2. (2010).

Culturing

The cultural conditions were similar to those of embryos excepting the incubation of materials under fully illuminated conditions, with fluorescence tube (Philips, India) having a photoperiod of 132/^{u} $Em^{-2}S^{-1}$.

Sub-culturing

After induction of calli in about 7 to 10 days, the calli aseptically transferred onto suitable media for proliferation and this procedure is repeated up to 12 passages in some of the hybrids.

Hardening of seedlings

Since seedlings are grown asceptically under controlled environment to ensure their survival, it is necessary to gradually acclimatize them before transferring them into soil. The culture tubes with seedlings were first transferred to normal room temperature (30 to 35°C and kept for two days. After removing the cotton plugs, the semisolid medium around the seedlings was washed gently under a slow jet of running water. Seedlings were left in glass tray containing tap water for 5 days to encourage initiation of new roots. They were then transferred to liquid growth medium and was allowed to grow for one week.

Transfer of seedlings to soil

Finally, plants were transferred to shallow pan pots containing sterilized, well puddle soil and kept in green house for 3 weeks. Later, plants were transferred to the earthen pot or to the field directly.

RESULTS

Two basal media, MS and N_6 were tested for callusing efficiency of the cultured panicle primordia of *O. sativa x O. brachyantha* hybrid. Two levels of auxins (2, 4-D and NAA) and one cytokinin (KIN) were used in several combinations. The results of the various combinations attempts along with their response are summarised in Table 1.

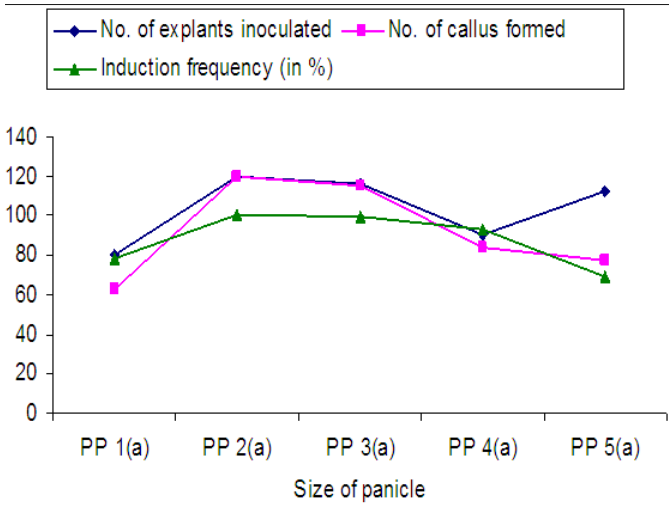

Figure 1. Effect of size of explants on callus induction.

Figure 2. Callus induction from immature inflorescences.

When the level of KIN (0.5 mg L^{-1}) was constant with varying levels of 2, 4-D, the callus induction frequency was high (54%) at 2.0 mg L^{-1} of 2, 4-D with Murashige and Skoog (MS) medium, whereas in N_6 medium the response was highest (60%) when 2.5 mg L^{-1} of 2,4-D was supplemented. In both the media, the callusing frequency was low at lower levels of 2, 4-D (Table 1). However, peak callusing period was delayed and proportion of embryogenic calli obtained was low in N_6 medium when compared to MS medium (Figures 1 and 2). Interestingly, when the level of KIN was increased to 1.0 mg L^{-1} and 2, 4-D being tested under similar concentrations as earlier, it was observed that, 100% callusing was obtained in the MS medium (Figure 2) containing 2.0 mg l^{-1} of 2,4-D, while at the same concentrations, the response in N_6 medium was comparatively low (74%). Conversely both in MS and N_6 media, the peak callusing period were much less (7 and 9 days, respectively). However, proportion of embryogenic calli was as high as 90% under MS medium, while it was only 56% in N_6 medium (in both the medium the callus induction was reduced at higher concentration of 2,4-D). Observation on the effect of 2, 4-D at a fixed level of NAA (1.0 mg l^{-1}) revealed very low frequency of callus induction in both MS and N_6 media at all concentration tested. When the concentration of NAA was inceased to 2.0 mg l^{-1}, there was no substantial increase callus induction frequency over that of 1.0 mg I-1 of NAA and the recovery of embryogenic calli was also less under both the media.

The period of callusing was generally increased in N_6 medium while there was no significant increase or decrease in the case of MS medium. The interaction of 2,4-D with fixed levels of NAA (1.0 mg l^{-1}) and KIN (1.0 mg I-1) revealed that, maximum induction frequencies of 100 and 88% respectively could be obtained quickly (7 to 8 days) when 2.0 mg l^{-1} of 2,4-D was supplemented to MS and N_6 media. However, the proportion of embryogenic calli was high (94%) in MS medium and low (66%) in N_6 medium. To identify suitable size of the explants for maximum callusing in *O. sativa/O. brachyantha* , F_1s of 5 different sizes were chosen which ranged from 0.5 to 2.0 cm and above with a size increment of 0.5 cm per each class. The immature inflorescence bits were inoculated onto MS medium with 2.0 mg l^{-1}/NAA and 1.0 mg l^{-1}/KIN, which was standardised earlier. The results obtained are presented in (Tables 1 and 2).

Of the five different sizes of panicle primordia cultured, maximum callus induction (100%) was noticed when the size ranged from 0.5 to 1.0 cm closely followed by the panicle primordia size ranging from 1.0 to 1.5 cm (99.14%). However, when the panicle primordia is larger than 2.0 cm or smaller than 0.5 cm, the response was very low (78.75 and 68.75% respectively). The effect duration of continuous sub-culturing and their ability to proliferate and redifferentiate was investigated in the calli of *sativa cv. Savitri* × *O. brachyantha* cross. Continuous subcultures at an interval of one month till 12 months are presented in Table 3 (Figures 3). It was observed that, the frequency of redifferentiation was high up to six subculture and started declining thereafter. Highest frequency (93.24%) of redifferentiation was recorded after fifth sub-culture and remained high as 58.82% even after 12^{th} sub-culture.

It was observed that, the frequency of redifferentiation was high up to third subculture and started declining thereafter. Highest frequency (90%) of redifferentiation was recorded after third subculture.

Table 2. Effect of size of explants on callus induction efficiency of immature inflorescence.

Primordial panicle (*)	Number of explants inoculated	Number of callus formed	Induction frequency (in %)	Peak callusing period (days)
PP 1(a)	80	63	78.75	6
PP 2(a)	120	120	100.00	5-7
PP 3(a)	116	115	99.14	5-8
PP 4(a)	90	84	93.33	12
PP 5(a)	112	77	68.75	16

*PP1 = 0.5 cm, PP2 = 0.5-1.0 cm, PP3 =1.0-1.5 cm, PP4 =1.5-2.0 cm, PP5 = above 2.0 cm, (a) = explants were cultured on MS+2.0 mg 2, 4-D + 1.0 mg KIN + 2.0 mg NAA per litre.

Table 3. Effect of subculturing duration on differentiation of calli.

Passage number	Number of Calli transferred	Number of calli redifferentiated	Percentage of redifferentiation
I	40	35	87.5
II	45	35	77.77
III	40	36	90.00
IV	50	40	80.00
V	60	56	93.24
VI	60	52	86.58
VII	40	24	60.00
VIII	50	30	68.18
IX	44	30	45.00
X	40	18	45.00
XI	40	18	45.00
XII	34	20	58.82

DISCUSSION

In the present study, among the wide cross hybrids, only two of the crosses, *O. sativa x O. brachyantha* showed complete sterility and therefore, suitable method was to be adopted for alien gene recombination. From these, one cross, *O. sativa* (*cv. Savitri*) x *O.brachyantha* was selected to study whether somatic cell culture could be helpful in realizing alien genome/chromosome recombination into cultivated rice. Exploration of somaclonal variation for crop improvement has been well demonstrated in rice (Fujiwara and Ojima, 1955; Amemiya et al., 1956; Furuhashi and Yatazawa, 1964; Kawata and Ishihara, 1968; Tamura, 1968). All these authors have used immature embryos, excised roots, stem nodes and intact roots where exogeneous application of 24-D stimulated the production of callus capable of indefinite growth. The advantage of somaclonal variation is that, a wide range of variability with regard to some qualitive and quantitative traits have been observed (Sun et al., 1983; Zhang and Chu, 1984). Zhang and Chu (1986), Indra and Krishnaveni (2009) and Croughan et al. (1986) advocated that, somaclones offer a potentially useful source of genetic variability for varietal improvement in rice. It has also been demonstrated in other cereals and millets (George and Eapen, 1990) that, the spectrum of variability is more in young inflorescence culture than from culture of other explants such as stems seeds and roots (Chen et al., 1985).

In the present study, since all the wide cross hybrids involving wild species having other than AA genomes are sterile and seed progeny from F1s could not be obtained, it was obligatory to use young inflorescence, here in referred to as prirnordial panicle are used as explants source for exploitation of somaclonal variation. Literatures on use of somaclonal variation of realization of recombinants with alien chromosomes are very few. However, Scowcroft et al. (1985) from their experience in wheat and other cereals used wide crosses, *Lolium perenne x L. Lumtiflorum, L. Multiflorum x Festuca arundinaceal, H. 5xplan x S. 5xplan, T. Creassum x H. 5xplan, Saccharum x Zea* and Triticale for somatic cell culture for alien gene introgression. They believed that many of the variatations are due to mutation. Brar and Khush (1986) opined that, somaclonal variation seems to be promising for introgression of alien genes into commercial cultivars. This would be particularly important in wide crosses where chromosomes do not pair. They suggested that, explants of wide crosses, (F1s)

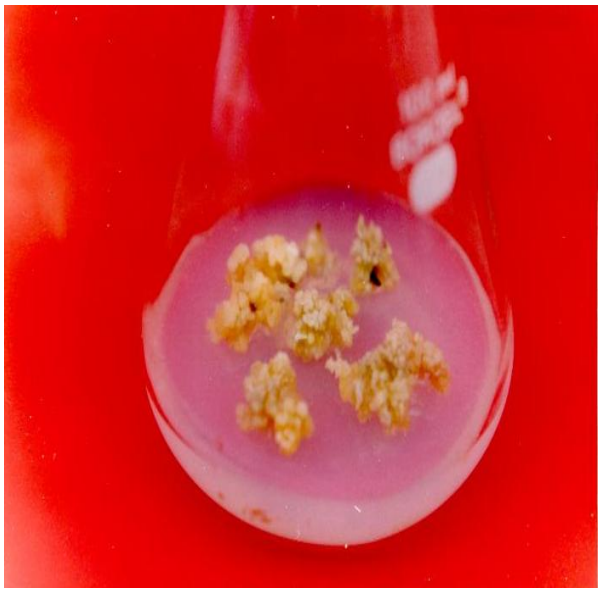

Figure 3. Calli on subculture medium

chromosome addition or substitution lines may enhance the frequency of genetic changes between alien and cultivated genomes. Orton (1980) reported the realization of somaclonal variation from tissue culture in *Hordeum vulgare x H. Jubatum* (a sterile hybrid) where enhanced bibalent formation was there as compared to the original hybrid which was asynaptic. A number of regenerants from that were haploids and Hordeum type. Two of the five haploids exmined expressed a few *H. Jubetum* isozyme bands indicating that some interspecific exchange might have occurred prior to chromosome elimination (Orton, 1980).

Recently, Sudhakar et al. (2009) and Lapitan et al. (1984) obtained regenerants with high degree of chromosome explants66 changes through somatic tissue culture from amphidiploids of wheat x rye hybrids. The karyotype analysis by C-banding of the 10-amphidiploids showed in 3 wheat x rye, in 1 wheat x wheat, translocations, in 7 delitions, in 5 heterochromatin amplification of rye chromosome. It is evident from the foregoing explants what somaclonal variation is one of the useful means to obtain alien gene introgression in cereals and particularly of interest in rice alien gene introgression through wide hybridization. Somaclonal variation using suitable explants could be exploited in three different ways:

(1) Organised plant tissues from plantlets in suitable media which usually contain plant hormones such as auxins and cytokinins. These plantlets are genetically identical with the parents and mutations are relatively rare and if present, as in some species such as potatoes, may be due to cytoplasmic factors or transposons.
(2) The second method is based on the formation of callus tissues and its subsequent utilization for organogenesis and plantlet formation. This method is, however, relatively more difficult than the method using organised tissues. This method is, however, relatively more difficult than the method using organised tissues. This method has been used less frequently but used in some cases as in oil palm. Chromosomal abnormalities occur often, especially on repeated sub-culture of the callus tissue prior to induction of organogenesis. This method is more useful for mutation programmes. The economy in space, time and labour compared to seed mutation makes mutation of the callus culture potentially one of the most attractive methods for the production desired strains for many biotic and abiotic stress and physiological attributes (Jagannathan, 1984).
(3) The third method is also based on initial callus induction from which embryoids and then plantlets are obtained. This method has been so far found applicable to very few species. But the large number of embryoids formed, the development of healthy root system similar to those of seed raised plants makes it an attractive method for exploitation of somaclonal variation. In the present study, since the interest is on induction of chromosome pairing and other abnormalities and mutation, the above second and third ways were effective when F1 primordial panicle was used as explants. Since the advantage of somaclonal variation is the capacity of the tissue to produce callus which yield sufficient variations, it is necessary to have the standardization of media and duration of culture and size of explants. It was observed that, when panicle is at a very young primordial stage where the size is about 0.5 to 1.5 cm, it takes hardly 5 to 7 days for callus initiation. Of the two basic media tested, MS medium supplemented with 2-4-D NAA and kinetin (2 mg L^{-1} 2 mg L^{-1} and 1 mgL^{-1}) was comparatively better for callus growth and proliferation. In this medium callus could be sub-cultured continuously for 12 passages (12 months) without loss of totipotency. However, peak regeneration was between 6 to 8 subculture stages. The present study was to develop an effective protocol for optimum callus induction, duration of culture and size of explants of primordial panicle of inter-genomic hybrid of *O. sativa/O. brachyantha*. The Murashige and Skoog (MS) medium and Chu's N_6 medium supplemented with 2-4-D, NAA and kinetin (2mg L^{-1} 2mg L^{-1} and 1mg L^{-1}) was better for callus growth and proliferation and callus was sub-cultured continuously for 12 passages (12 months) without loss of totipotency. Results indicated that, good plant regeneration could be best effected with 1.5 to 2.0 mg L^{-1} of KIN at fixed level of NAA (at 0.5 mg L^{-1}). These variations via tissue culture will be helpful in alien gene transfer especially in incompatible species of rice in wide hybridization.

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