Effect of sucrose on callus induction and green plantlet regeneration in anther culture of *Indica x Indica* rice

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**Abstract.** Anther culture is an important biotechnological tool. By rice anther culture homozygous pure lines in the form of doubled haploid (DH) plants can be produced within a year as compared to the long inbreeding method, which might take 8-10 years. Application of anther culture technique in breeding and genetics research is limited due to the very low regeneration frequency of anthers of rice in general, and *indica* cultivars in particular. Therefore, the successful use of the technique depends on the adequate production of DH plants for selection and field evaluation. Effect of various concentrations of sucrose in callus induction media was investigated on callus induction and regeneration of green plantlets from anther culture of several F1s derived from *indica x indica* crosses. Cold pretreated anthers of five genotype of anther donor plants (F1s) at 5 °C for 8 days were cultured on N6 callus induction medium containing sucrose at a concentration of 6.0%, 6.5%, and 7.0%. Results revealed that all genotypes showed similar response to callus induction, but a significantly different responses to plant regeneration depended on sucrose level in the callus induction media. Two genotypes, i.e. IR78788 / Inpara 5 and Dendang/Inpari 30 only regenerated albino plantlets. In this experiment, 6.5% sucrose compared to two other sucrose treatments was suitable for inducing high frequency callus induction and high green plantlet regeneration in all genotypes. The high concentration of sucrose (7.0%) in the culture medium not only resulted in a decrease in the number and percentage of callus formation but also decreased the regeneration of green plantlets.

1. **Introduction**

Rice (*Oryza sativa* L.) is a highly self-pollinated plant. It normally needs more than 8 cycles of selfing and subsequent selection to obtain pure lines of interest prior to 3-5 more years of field evaluation to develop a variety [1]. The production of pure lines using doubled haploids (DH) has several advantages over conventional methods. Using DH production systems, pure lines that are highly homozygous is achieved in one generation. The DHs can be readily selected as materials suitable for variety development in rice breeding programs, thus eliminating the need for several generations of self-pollination [2, 3, 4]. Several in-vitro techniques (e.g. anther culture, pollen or microspore culture, and ovule culture, etc.) have been developed for the production of DHs. The techniques have been used in breeding programs of many plant species, but until recently, anther culture is the most
effective and widely used in-vitro technique to obtain DH plants [5, 6, 7, 8]. The anther culture method relies on the ability of microspores or immature pollen grains to convert their developmental pathway from gametophytic leading to mature pollen grain, to sporophytic resulting in androgenesis. Androgenesis in rice anther culture is a process where cell division at a haploid level followed by the formation of calluses which later differentiate into plants [9, 2]. In rice anther culture, a haploid set of chromosomes in microspores also becomes spontaneously doubled under suitable culture conditions [5]. However, rice anther culture response is species and genotype specific, thus successful use of anther culture in varietal development depends on the efficient production of adequate numbers of DH plants for selection and field evaluation [10]. The anthers of hybrid (F1) progeny are excellent breeding material for raising pollen-derived homozygous plants or DH in which complementary parental characteristics are combined in one generation [11]. The main limiting factor in using this technique to accelerate rice breeding in Indonesia is due to the utilizations of subspecies indica as parents in parental mating. In general, indica cultivars of rice exhibit low regeneration frequency or poorer androgenic response than the japonica cultivars [12, 13, 14].

Initiation of androgenesis depended upon the chemical composition of the media including the source of carbon [5, 15]. A carbohydrate source is the most essential in anther culture because of its osmotic and nutritional effects during embryogenesis or organogenesis. In general, sucrose is considered as an important carbon and energy source in plant tissue culture as it is the most common carbohydrate found in phloem sap and involved in controlling various development processes [16]. Sucrose concentration of 3.0% is the most commonly used carbohydrate source in tissue culture [17]. However, in anther culture, a higher concentration of sucrose (4-6%) has been used as a carbon source in callus induction medium to regulate the osmotic pressure, stimulate dedifferentiation during growth induction and to prevent callus formation from somatic anther tissue with respect to the number of embryo-like structures [18, 19]. Since the success of indica rice anther culture is depend firstly on the calli formation from microspore, thus an appropriate concentration of sucrose is needed in the callus induction medium to promote callus formation and subsequent plant regeneration from callus. Therefore, the objective of this research was to investigate the effect of various concentrations of sucrose on callus induction and plantlet regeneration from anther culture of several genotypes of anther donors (F1s) derived from indica x indica crosses.

2. Materials and Methods

2.1. Materials

Anther donor plants used in this research were five F1s, each derived from a single cross between indica rice as parents of interest, i.e. elite breeding rice line IR78788 or high yielding variety, i.e. Dendang, Inpari 29, Inpari 30, and Inpara 5. These donor plants were planted in the greenhouse until the booting stage when young panicles were collected. Panicles were cold pretreated at 5°C for 8 days prior to anther culture. Callus induction medium was N6 2.0 mg NAA.L-1 + 0.5 mg Kinetin.L-1 + 10^-3 M putrescine, while regeneration medium was MS + 0.5 mg NAA.L-1 + 2.0 mg Kinetin.L-1 + 10^-3 M putrescine + 3.0% sucrose [14]. Phytagel® (3 g/L) was used as a solidifying agent in both media.

2.2. Methods

The experiment was performed using a Randomized Factorial Design consisting of 3 replications. The treatments used were five genotype of anther donor plants (F1s), i.e. F1: IR78788 / Dendang, F1: IR78788 / Inpari 29, F1: IR78788 / Inpari 5, F1: Inpari 30 / Dendang and F1: Dendang / Inpari 30; and three level of sucrose concentration added to the callus induction media, i.e. 6.0% (control), 6.5%, and 7.0%. Each experimental unit was one petridish containing anthers from 25 spikelets. Rice anther culture method followed the protocol of Dewi et al. [20].

Observation was conducted on callus initiation period, number of anther-derived calli produced, number of calli producing plantlet, number of calli forming green plantlets, number of calli forming albino plantlets, number of calli-derived plantlets, number of green and albino plantlets, number of
survivor green plants, and number of first-generation of DH (DH0) plants. Observation on anther response to callus induction was terminated at 60 days after anther plating. Statistical analysis was performed using variance analysis. The differences between treatments were tested by Duncan Multiple Range Test (DMRT). Data analysis was carried out using the Statistical Tool for Agricultural Research (STAR) version 2.0.1.

3. Results and Discussion

3.1. Effect of sucrose on callus induction

The callus initiation period is significantly affected by the interaction between the genotypes of anther donor and sucrose concentrations (Table 1). The onset of callus induction in this experiment started 4-6 weeks after anthers were plated (DAP). The longest day for first callus formation (CIP) took 56.4 DAP in IR78788 / Inpara 5 followed by IR78788 / Inpari 29 at 51.2 DAP. In anther culture the splitting of the anther lobes to release the microspores into callus induction medium is important as the first step to androgenesis. The mechanism that regulates the splitting of anther lobes heavily depends on the osmotic pressure caused by high levels of the carbon source in the callus induction media [5]. In this research, 6.0-7.0% of sucrose in callus induction medium is enough to cause the osmotic pressure needed for splitting the anther lobes.

Table 1. Effect of sucrose to callus induction in rice anther culture of F1 indica x indica crosses

| Genotypes (F1)       | [Sucrose] | CIP (DAP) | CP       | CP * (%) |
|----------------------|-----------|-----------|----------|----------|
| IR78788/Dendang      | 6.0       | 40.10 def A | 7.25 cde A | 4.83     |
|                      | 6.5       | 41.45 cde A | 8.70 bcd A | 5.80     |
|                      | 7.0       | 39.20 def A | 4.15 de B  | 2.77     |
| IR78788/Inpari 29    | 6.0       | 44.25 cd B  | 2.15 e B   | 1.43     |
|                      | 6.5       | 46.15 c B   | 6.15 cde A | 4.10     |
|                      | 7.0       | 51.20 b A   | 2.30 e B   | 1.53     |
| IR78788/Inpara 5     | 6.0       | 46.35 c B   | 2.15 e B   | 1.43     |
|                      | 6.5       | 52.45 ab A  | 3.80 de A  | 2.53     |
|                      | 7.0       | 56.45 a A   | 1.50 e B   | 1.00     |
| Inpari 30/Dendang    | 6.0       | 44.05 cd A  | 4.25 de B  | 2.83     |
|                      | 6.5       | 39.40 def B | 13.60 ab A | 9.07     |
|                      | 7.0       | 40.90 de AB | 10.80 bc A | 7.20     |
| Dendang/Inpari 30    | 6.0       | 35.60 f B   | 8.95 bcd B | 5.97     |
|                      | 6.5       | 37.25 ef AB | 16.70 a A  | 11.13    |
|                      | 7.0       | 40.45 def A | 6.15 cde B | 4.10     |

Notes: CIP= callus initiation period; CP= no. of anther-derived calli; DAP =days after anther plating. Number in the same column followed by the same lower case letter are not significantly different by DMRT at P < 0.05. Numbers in the same column at each genotype followed by the same capital letter are not significantly different by DMRT at P < 0.05. * no statistical analysis.

Interaction between genotypes and sucrose concentrations made a considerable variation for pollen callusing among the anther donor genotypes (Table 1). The genotypes could produce more than one callus from pollen in one anther depending upon the F1’s genotype. The number of anther-derived calli (CP) are significantly affected by sucrose concentration (Table 1). In general, 6.5% of sucrose gave a significant increase in CP. However, the ability of immature pollen grain to proliferate into a callus was determined by the genotype of F1s (19). F1 derived from Dendang/Inpari 30 gave the highest number of callus (11.3%) when 6.5% sucrose was added to callus induction media. Compared
to the addition of 6.0% and 6.5% sucrose, the addition of 7.0% sucrose gave a low percentage of CP in all genotypes, except for Inpari 30/Dendang. This may be due to the termination of cell development by a high amount of sucrose [21]. Therefore, in anther culture all calli as small as 2 mm in diameter should be immediately transfer into the regeneration medium to avoid retardation in callus development [22].

3.2. Plant regeneration from anther-derived calli
The number and rate of callus producing plantlet (CPP) among the anther donor genotypes were significantly affected by genotypes and source of calli (Table 2). The addition of 6.5% sucrose into callus induction medium significantly increased CPP in regeneration medium, however the rate of CPP was decreased significantly when callus came from a medium with 7.0% sucrose. The best calli producing green plantlet (CPG) also came from callus induction media with 6.5% sucrose. There were calli from two genotypes, i.e. IR78788 / Inpara 5 and Dendang/Inpari 30, produced only albino plantlet (CPA) when they came from callus induction media with 6.0 (control) and 7.0% sucrose (Table 2).

Table 2. Rate of calli producing plantlet in rice anther culture of F1 indica x indica crosses.

| Genotypes (F1) | Source of calli from [Sucrose] | CPP | CPG | CPA | CPP (%) a | CPG (%) a | CPA (%) a |
|----------------|-------------------------------|-----|-----|-----|-----------|-----------|-----------|
| IR78788 / Dendang | 6.0 | 1.75bc AB | 0.25de B | 1.50bcd A | 24.14 | 3.45 | 20.69 |
|                | 6.5 | 2.35b A | 0.75bc A | 1.60bc A | 27.01 | 8.62 | 18.39 |
|                | 7.0 | 0.70de B | 0.10e B | 0.60efg B | 16.87 | 2.41 | 14.46 |
| IR78788 / Inpari 29 | 6.0 | 0.95cde B | 0.10e B | 0.85c-g B | 44.19 | 4.65 | 39.53 |
|                | 6.5 | 2.45b A | 0.50cd A | 1.95ab A | 39.84 | 8.13 | 31.71 |
|                | 7.0 | 0.70de B | 0.20de B | 0.50efg B | 30.43 | 8.70 | 21.74 |
| IR78788 / Inpara 5 | 6.0 | 0.30e B | 0.00e B | 0.30fg AB | 13.95 | 0.00 | 13.95 |
|                | 6.5 | 1.55bcd A | 0.95ab A | 0.60efg A | 40.79 | 25.00 | 15.79 |
|                | 7.0 | 0.05e B | 0.00e B | 0.05g B | 3.33 | 0.00 | 3.33 |
| Inpari 30 / Dendang | 6.0 | 0.60de B | 0.05e B | 0.55efg B | 14.12 | 1.18 | 12.94 |
|                | 6.5 | 2.25b A | 1.15a A | 1.10c-f A | 16.54 | 8.46 | 8.09 |
|                | 7.0 | 0.80de B | 0.10e B | 0.70d-g AB | 7.41 | 0.93 | 6.48 |
| Dendang / Inpari 30 | 6.0 | 1.30cd B | 0.00e B | 1.30b-e B | 14.53 | 0.00 | 14.53 |
|                | 6.5 | 3.70a A | 1.05ab A | 2.65a A | 22.16 | 6.29 | 15.87 |
|                | 7.0 | 1.00cde B | 0.00e B | 1.00c-f B | 16.26 | 0.00 | 16.26 |

Notes: CPP: callus producing plantlet; CPG: callus producing green plantlet; CPA: callus producing albino plantlet. Numbers in the same column followed by the same lower case letter are not significantly different by DMRT at P < 0.05. Numbers in the same column at each genotype followed by the same capital letter are not significantly different by DMRT at P < 0.05. a no statistical analysis.

The number of regenerated plants significantly affected by genotypes and the source of calli (Table 3). The number of plantlet, number of green and albino plantlets regenerated were varied significantly depending upon the genotypes of the anther donor plants. In this research, all genotypes gave a considerable amount of plantlets, but the rate of albino plant (AP) regeneration was very high, ranged from 68.13% to 100%. Since a large number of albino plantlet is always produced in anther culture of cereal, thus to effectively use androgenetic doubled-haploids in breeding programs, it is essential to increase green plantlet (GP) production [14, 2, 23]. Calli from a medium with 6.5% sucrose increase green plant regeneration in all genotypes including those genotypes, i.e. IR78788 / Inpara 5 and Dendang / Inpari 30, that did not produce any green plant at 6% (Control) and 7% sucrose. Another
interesting finding is 6.5% sucrose in callus induction medium also minimized the frequency of albino plants from anther-derived calli compared to control (6.0% sucrose) and 7% sucrose (Table 3). Like in any other research involving anther culture, the major constraints in incorporating this technique into a breeding program seem to be the limited morphogenetic potential of anther-derived calli and a higher percentage of regenerated albino plants. The basic cause for albino plant production is the breakage of DNA in plastids and nuclei [24].

Table 3. Effect of sucrose on plant regeneration in rice anther culture of F1 *indica* × *indica* crosses.

| Genotypes (F1) | Source of calli from (Sucrose) | Regenerated Plants |
|---------------|-------------------------------|--------------------|
|               | GP          | AP      | GP+AP  | GP (%)a | AP (%)a |
| IR78788 / Dendang | 6.0%      | 0.45cd B | 5.65bcd A | 6.10bc A | 7.38 | 92.62 |
|               | 6.5%      | 1.90a A  | 6.05abc A | 7.95b A  | 23.90 | 76.10 |
|               | 7.0%      | 0.15cd B | 1.90efg B | 2.05def B | 7.32 | 92.68 |
| IR78788 / Inpari 29 | 6.0%      | 0.25cd B | 2.70efg B | 2.95b B  | 8.47 | 91.53 |
|               | 6.5%      | 1.00bc A | 7.15ab A  | 8.15b A  | 12.27 | 87.73 |
|               | 7.0%      | 0.55cd AB| 1.85efg B | 2.40def B | 22.92 | 77.08 |
| IR78788 / Inpara 5 | 6.0%      | 0.00d B  | 1.00efg B | 1.00ef B | 0.00 | 100.00 |
|               | 6.5%      | 1.45ab A | 3.10d-g A | 4.55cd A | 31.87 | 68.13 |
|               | 7.0%      | 0.00d B  | 0.20g B   | 0.20f B  | 0.00 | 100.00 |
| Inpari 30 / Dendang | 6.0%      | 0.05d B  | 1.90efg B | 1.95def B | 2.56 | 97.44 |
|               | 6.5%      | 1.80ab A | 4.15cede A | 5.95bc A | 30.25 | 69.75 |
|               | 7.0%      | 0.25cd B | 2.40efg B | 2.65def B | 9.43 | 90.57 |
| Dendang / Inpari 30 | 6.0%      | 0.00d B  | 3.95c-f B | 3.95cde B | 0.00 | 100.00 |
|               | 6.5%      | 2.25a A  | 8.80a A   | 11.05a A | 20.36 | 79.64 |
|               | 7.0%      | 0.00d B  | 2.50efg B | 2.50def B | 0.00 | 100.00 |

Notes: GP= green plantlet; AP= albino plantlet. Numbers in the same column followed by the same lower case letter are not significantly different by DMRT at P < 0.05. Numbers in the same column at each genotype followed by the same capital letter are not significantly different by DMRT at P < 0.05. a no statistical analysis.

3.3. Anther culture efficiency and doubled-haploid production

Interaction between genotypes and sucrose concentration determined the anther culture efficiency (ACE) of *indica* × *indica* crosses (Table 4). According to Zhang [25] the ACE can be represented by the rate of green plantlets (GP) produced in regard to the number of anther-derived calli produced (CP) or in regard to number of anther plated (ATP). Thus, green plantlet regeneration depends heavily on a responsive genotype. Different responses to anther culture were observed among indica cultivars and its hybrid, and even good calli induction may result in predominant albino populations [14, 5, 22]. In this research compared to 6% sucrose (control), GP/CP and GP/AP in all genotypes were increase by 6.5% sucrose, while 7% sucrose only increase GP/CP and GP/AP in IR78788/Inpari 29 and Inpari 30/Dendang. For certain genotypes such as IR78788/Inpara 5 and Dendang/Inpari 30 only the addition of 6.5% sucrose to callus induction medium successfully induced green plant regeneration from 0.00% to 38.16% and 13.47%, respectively. This indicates that the addition of 6.5% sucrose into the callus induction medium has an influence on the morphogenetic competence of the induced callus, determining its regeneration capability.

From this experiment, all green plantlets (202 green plantlets) were acclimatized (NAP) and 156 surviving plants (NSP) were planted in the greenhouse (Table 4). Spontaneous doubled-haploid (DH0) plants obtained from this experiment were 49 lines or approximately 31.4% of the total survivor plants. Germana [5] reported that spontaneous chromosome doubling (endoreduplication) in anther
culture can be achieved up to 50%. In this research, DH0s were mainly produced from calli-derived green plantlets of 6.5% sucrose. Dendang / Inpari 30 produced the highest number of DH0 lines (Table 4).

Table 4. Effect of sucrose on anther culture efficiency and doubled haploid plant production in rice anther culture of F1 indica x indica crosses

| Genotype (F1) | [Sucrose] | GP/CP (%) | GP/ATP (%) | NAP | NSP | DH0 | NSP (%) | DH0 (%) |
|---------------|-----------|-----------|------------|-----|-----|-----|---------|---------|
| IR78788 / Dendang | 6.0% | 6.21 | 0.30 | 9 | 5 | 0 | 56 | 0 |
| | 6.5% | 21.84 | 1.27 | 38 | 19 | 2 | 50 | 10.5 |
| | 7.0% | 3.61 | 0.10 | 3 | 0 | 0 | 0 | 0 |
| IR78788 / Inpari 29 | 6.0% | 11.63 | 0.17 | 5 | 3 | 1 | 60 | 33.3 |
| | 6.5% | 16.26 | 0.67 | 20 | 18 | 2 | 90 | 11.1 |
| | 7.0% | 23.91 | 0.37 | 11 | 7 | 0 | 64 | 0 |
| IR78788 / Inpara 5 | 6.0% | 0.00 | 0.00 | 0 | 0 | 0 | - | - |
| | 6.5% | 38.16 | 0.97 | 29 | 28 | 5 | 97 | 17.9 |
| | 7.0% | 0.00 | 0.00 | 0 | 0 | 0 | - | - |
| Inpari 30 / Dendang | 6.0% | 1.18 | 0.03 | 1 | 0 | 0 | 0 | - |
| | 6.5% | 13.24 | 1.20 | 36 | 32 | 6 | 89 | 18.8 |
| | 7.0% | 2.31 | 0.17 | 5 | 3 | 1 | 60 | 33.3 |
| Dendang / Inpari 30 | 6.0% | 0.00 | 0.00 | 0 | 0 | 0 | - | - |
| | 6.5% | 13.47 | 1.50 | 45 | 41 | 32 | 91 | 78 |
| | 7.0% | 0.00 | 0.00 | 0 | 0 | 0 | - | - |

Note: GP= no. of green plant; CP= no. of anther-derived calli; ATP = no. of anther plated; NAP= Number of acclimatized plantlet; NSP= Number of plantlet survived from hardening; DH0= first generation of dihaploid plant; *no statistical analysis..

4. Conclusions

Interaction between genotypes and sucrose concentrations made a considerable variation for pollen callusing among the anther donor genotypes. The high concentration of sucrose (6.0%, 6.5% and 7.0%) in callus induction medium promotes the proliferation of callus and subsequent plantlets development. However, there were genotypes that did not produce any calli-derived green plantlet, i.e. IR78788 / Inpara 5 and Dendang / Inpari 30, at 6% (control) and 7% sucrose. In general, the most efficient concentration of sucrose can be added to callus induction medium was 6.5%, higher sucrose concentration (7.0%) may lead to a decrease in the regenerative competence of anther-derived calli. The addition of 6.5% sucrose in callus induction medium also minimized the frequency of albino plants from anther-derived calli compared to control (6.0% sucrose) and 7% sucrose.

Author Contributions

All authors contributed equally to the manuscript. ISD and BSP sought funding, planning and supervised the work. HS performed the experiment and processed the experimental data. ISD drafted the manuscript and designed the tables. All authors performed the analysis, discussed, interpreted the final results and commented on the manuscript.
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