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# Comparing in vitro plant regeneration ability of *Oryza sativa* L. cv. Fujisaka 5 and *Brachiaria decumbens* from embryogenic callus

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**Abstract.** *Daud Z, Osman AS, Jelodar NB, Chan L-K. 2022. Comparing in vitro plant regeneration ability of* Oryza sativa *L. cv. Fujisaka 5 and* Brachiaria decumbens *from embryogenic callus. Cell Biol Dev 7: 20-27.* The aim of this study was to compare the plant regeneration ability of *Oryza sativa* L. cv Fujisaka 5, a cold resistance japonica rice and *Brachiaria decumbens* Stapf, a tropical savanna grass, using embryogenic calli. The plant regeneration ability of both species has not been reported elsewhere. Friable calli were induced from the *O. sativa* Fujisaka 5 seeds on MS medium supplemented with 2.0 $mgL^{-1}$ 2,4-D. While embryogenic calli were produced from *B. decumbens* seeds using a similar culture medium and remained embryogenic even after frequent subculturing. The friable calli of *O. sativa* Fujisaka 5 became embryogenic (88.2-97.7%) when they were subcultured onto MS medium containing 2,4-D and kinetin or BAP and NAA (0.5-1.0 $mgL^{-1}$) for four weeks. When the induced embryogenic calli (0.5 g) of both species were subcultured onto MS without plant growth regulators, plantlets were generated after one to two weeks with the formation of 2-3 plantlets for *O. sativa* Fujisaka 5 and 4-5 plantlets for *B. decumbens* per 0.5 g calli. The present study proved that plant regeneration of *B. decumbens* could be accomplished via direct somatic embryogenesis, which involved two stages: initiation of somatic embryos from germinating seeds on MS medium supplemented with 2.0 $mgL^{-1}$ 2,4-D and plantlet regeneration achieved via transferring the somatic embryos onto MS medium without PGRs. Plantlets of *O. sativa* Fujisaka 5 were established via indirect somatic embryogenesis which involved three stages: induction of callus from germinating seeds on MS medium supplemented with 2.0 $mgL^{-1}$ 2,4-D, followed by the production of somatic embryos using MS medium containing 0.5-1.0 $mgL^{-1}$ 2,4-D and kinetin or BAP and NAA and finally plant regeneration by subculturing the somatic embryos onto MS medium without PGRs. These established plant regeneration protocols of *O. sativa* Fujisaka 5 and *B. decumbens* would be useful for future rice improvement research.

**Keywords:** *Brachiaria decumbens*, cold resistance japonica rice, *Oryza sativa* cv Fujisaka 5, somatic embryogenesis, tropical savanna grass

**Abbreviations**: 2,4-D: 2,4-Dichlorophenoxy Acetic Acid, BAP: 6-benzylaminipurine, NAA: 1-Naphthaleneacetic Acid, MS: Murashige and Skoog Medium (1962), PGRs: Plant Growth Regulators

## INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important grain crops providing a global food supply. It is cultivated mostly in Asia (90.6%), Africa (3.5%), the Americas (5.2%), and the balance of 0.7% in Europe and Oceania. Global rice production is projected to reach 582 million tons in 2029, and Asia will contribute much of the world's rice production (OECD/FAO 2020). With the estimated world population of 7.7 billion in 2019 and expected to reach 8.5 billion by 2030 (United Nations 2019), rice production must increase to meet the growing population's demand. Several biotic (diseases, insects) and abiotic (drought, salinity, iron, and aluminum toxicity) stresses are additional causes that reduce rice productivity. Hence, there is a continuous need to develop new rice varieties with higher yield potential and durable resistance to diseases, insects, and abiotic stresses through conventional and biotechnological means.

Rice is the staple food crop in Malaysia. The rice production in Malaysia only fulfills 70% of its rice requirements. The remaining 30% are imported, mainly from Thailand, Vietnam, and Pakistan (Fatah and Cramon-Taubadel 2017). Between 1964 and 2018, Malaysia released 49 rice varieties to increase the production yield with each release of new varieties (Sarena et al. 2019).

Gene transformation and somatic hybridization have been employed as tools for rice improvement. These techniques enable unlimited access to the gene pool by transferring desirable genes. The development of new rice hybrids or rice improvement of different cultivars has been successfully achieved via agrobacterium-mediated transformation, particle bombardment-mediated transformation and protoplast transformation techniques. The Golden rice which produces β-carotene is the successful end-product of these genetic engineering processes (Tan et al. 2017; Page et al. 2019; Poddar et al. 2020)

Hybrid Indica rice is the dominant rice in tropical and subtropical countries. In contrast, the breeding and promotion of hybrid japonica rice are limited, but the demand for japonica rice is continuously increasing in China (Zheng et al. 2020). Cui et al. (2020) reported that the demand and increasing planting of japonica rice varieties have increased for the past decades; hence, it is most likely to be the dominant grain production in China. For the past decade, research on japonica super rice varieties has been actively conducted in Jiangsu Province, China (Wang et al. 2017). The *O. sativa* Fujisaka 5, a cold resistance japonica rice, possessed more panicles and a higher yield than many other rice cultivars. It also matures fast and can be harvested after 111 days of planting, as Dai et al. (1990) reported. *Brachiaria decumbens* Stapf, a tropical and perennial savanna grass, was found to possess desirable genes such as disease and insect resistance, cold and flood tolerance, and high grain quality (Low 2015). These desirable genes of *B. decumbens* could be transferred into the *O. sativa* Fujisaka 5 for future rice improvement via genetic transformation and somatic hybridization techniques. A foreign gene from different plant species other than rice or even bacteria or viruses has been used to create a new variety of transgenic rice with important agronomic traits such as pest resistance, cold and drought tolerance, and resistance to other abiotic and biotic stress via different gene transformation techniques (Low et al. 2018).

Even though there is unlimited information on rice improvement research using various *in vitro* culture techniques, none of these studies were carried out on *O. sativa* Fujisaka 5 and *B. decumbens* except a report on protoplast-derived plants of *O. Sativa* Fujisaka 5 by Ogura et al. (1987). The lack of *in vitro* plant regeneration techniques is one of the main barriers to developing a new rice cultivar. Most of the failures in genetic transformation are mainly due to poor regeneration potential (Rahman et al. 2021). Plant regeneration ability is a prerequisite for a successful transformation process (Mostafiz and Wagiran 2018). The present study was conducted to compare the plant regeneration ability and establish a protocol for *O. sativa* Fujisaka 5 and *B. decumbens* from induced embryogenic callus. Therefore, to the best of the authors' knowledge, the in vitro plant regeneration ability and protocol have not been reported for *O. sativa* Fujisaka 5 and *B. decumbens.*

## MATERIALS AND METHODS

### Plant materials

Mature seeds of *O. sativa* Fujisaka 5 were obtained from the Malaysian Agriculture Department (MARDI) Gene Bank, Seberang Perai, Penang, Malaysia while the mature seeds of *B. decumbens* were obtained from The International Maize and Wheat Improvement Center (CIMMYT), Mexico.

### Establishment of aseptic seed explants

The selected healthy seeds of *O. sativa* Fujisaka 5 were de-husked, washed with mild detergent, and rinsed under running tap water for 30 minutes. The cleansed seeds were then surface sterilized with 70% ethanol for 10 minutes followed by 20% Clorox® (a commercial bleach containing 5.3% sodium hypochlorite, The Clorox Company, Oakland, California, USA) added with a few drops of Teepol (TEEPOL, Malaysia) for 30 minutes and rinsed three times with sterile distilled water (Zainah 2015). The surface-sterilized seeds were inoculated on gelled MS basal medium (Murashige and Skoog 1962) containing 3% sucrose. Furthermore, 10 seeds were used for each sample unit, and 10 sample units were used for the trial. The same procedure was applied to *B. decumbens* seeds. Both seed cultures were kept in a culture room under continuous lighting provided by cool white, fluorescent lights with a light intensity of 30-32.5 $\mu molm^{-2}s^{-1}$ at 25 ± 2°C temperature. The percentage of aseptic seeds established and seed germination were determined after 10 days of culture.

### Induction of callus

The aseptic seeds of *O. sativa* Fujisaka 5 and *B. decumbens* were cultured separately on MS medium supplemented with various concentrations (2-10 $mgL^{-1}$) of 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich (M) Sdn. Bhd., Subang Jaya, Malaysia). Next, 10 replicates were used for each medium treatment, and the experiment was repeated 3 (three) times. The cultures were kept in a culture room under continuous lighting provided by cool white fluorescent lights with a light intensity of 30-32.5 $\mu mol\ m^{-2}\ s^{-1}$ at 25 ± 2 °C temperature. After four weeks of culture, the amount of callus induced was determined using a digital scale (Denver Instrument XL-410, Chicago, IL). The best callus induction medium was determined based on the amount of callus produced. The induced calli were transferred to the selected callus proliferation medium (MS + 2 $mgL^{-1}$ 2,4-D) to multiply calli for five subculture cycles (four weeks/cycle). The type of callus produced for each species was then determined.

### Induction of embryogenic callus of *Oryza sativa* cv. Fujisaka 5

*Effect of MS strength*

Approximately 0.5 g of the friable callus of *O. sativa* Fujisaka 5 was inoculated onto different strengths of MS medium (full-strength, ½-strength, ¼-strength, and 1/5-strength) without any plant growth regulator (PGR). Full-strength MS medium contains a full concentration of mineral salts (macro and micronutrients) and organic vitamins of the MS medium while ½-strength, ¼-strength, and 1/5-strength, contain half, a quarter and one-fifth of its concentration respectively. The cultured calli were maintained under continuous light provided by cool white, fluorescent lights with an intensity of 32.5 $\mu Em^{-2}s^{-1}$ at 25 ± 2°C in the culture room. Eight replicates were used for each medium treatment. The experiment was repeated three times. The fresh embryogenic callus mass was determined using a digital weighing scale (Denver Instrument XL-410, Chicago, IL). The proliferation of the embryogenic calli was determined after four weeks of culture based on the growth index according to Godoy-Hernández and Vázquez-Flota (2012).

$$growth\ index = \frac{final\ fresh\ weight(g) - initial\ fresh\ weight(g)}{initial\ fresh\ weight(g)}$$

*Effect of 2,4-D and kinetin*

Approximately 0.5 g of the friable callus of *O. sativa* Fujisaka 5 were inoculated onto MS medium supplemented with different concentrations of 2,4-D (0, 0.5, and 1.0 $mgL^{-1}$) and kinetin (0, 0.5 and 1.0 $mgL^{-1}$) and maintained in the culture room under the similar condition as previous experiments. Eight sample units were used for each combination treatment, and the experiment was repeated three times. The embryogenic callus formation with its growth index and morphology was determined and recorded after four weeks of culture.

*Effect of BAP and NAA*

Approximately 0.5 g of friable callus of *O. sativa* Fujisaka 5 were inoculated onto MS medium supplemented with different concentrations of 1-Naphthaleneacetic acid (NAA) (0. 0.5 or 1.0 $mgL^{-1}$) and 6-benzylaminipurine (BAP) (0, 0.5 or 1.0 $mgL^{-1}$). Eight replicates were used for each medium tested, and the experiment was repeated three times. The growth index of the induced callus for each treatment was calculated, and the morphology and color of the embryogenic callus formed were also observed and recorded after four weeks of culture. All the cultures were incubated in the room under conditions similar to previous experiments.

**Plant regeneration ability study**

For *B. decumbens,* 0.5 g of the embryogenic callus, derived and proliferated on MS solid medium supplemented with 2.0 $mgL^{-1}$ 2,4-D, was transferred onto PGR-free MS medium. While for *O. sativa cv.* Fujisaka 5, 0.5 g of the embryogenic callus, derived from MS medium supplemented with a combination of 2,4-D and kinetin (0, 0.5, 1.0 $mgL^{-1}$), and combination of BAP and NAA (0, 0.5, 1.0 $mgL^{-1}$), were transferred onto PGR-free MS medium. The number of plantlets regenerated was determined after two weeks of cultures. All the cultures were placed in the room under conditions similar to previous experiments.

**Histology of *Oryza sativa* cv. Fujisaka 5 somatic embryos**

A histology study was done on the green embryogenic callus of *O. sativa* Fujisaka 5 since it can generate normal plantlet formation. The green somatic embryos were removed from the culture medium and fixed immediately in a fixing solution containing 5% formaldehyde, 5% acetic acid and 90% ethanol (FAA) for 24 hours. The fixed tissues were then passed through a series of alcohol solutions, starting with 50% ethanol/tetra butyl alcohol (TBA) (ethanol 95%: absolute TBA: water = 4: 1: 5) and finally treated with absolute ethanol/TBA (ethanol 95%: absolute TBA = 2.5: 7.5). After which the samples were immersed in a mixture solution of TBA and liquid wax with ratio 1: 1 at 60-62 ºC for 24 hours. The solidified wax blocks containing the tissue samples were sliced into 10 µm thickness slices using a rotary microtome (Leica, Germany). The double-stained standard technique was used for the preparation of permanent slides. Each sliced section was placed on a glass slide and treated with a few drops of safranin. Next, a few drops of 95% ethanol were added to remove the excess safranin. Then, it was followed by adding a few drops of Fast Green and 95% ethanol, respectively. Finally, one drop of xylene was added to the section. The sliced section was then covered with a glass cover slip and sealed with Shandon Mount (Shan don, USA) as a mounting agent. The prepared slides were observed under a light microscope and documented (Olympus BX 50, Japan).

**Data analysis**

The establishment of aseptic seeds and their germination rate for *O. sativa* Fujisaka 5 and *B. decumbens* were compared using the Student t-test. The studies for induction of callus for each species, and the effect of MS strength on induction of embryogenic callus of *O. sativa* Fujisaka were carried out using a completely randomized design. These data were analyzed using one-way ANOVA, and the best callus induction medium or the best MS strength was selected after comparison of the mean using the Tukey´s test at $p \leq 0.05$ with the aid of SPSS version 20.0. The experiments to determine the effect of PGRs on the induction of embryogenic callus of *O. sativa* Fujisaka 5 were carried out using a Complete Randomized Block Design (CRBD). The data collected were then analyzed using two-way ANOVA, and the significant difference of means was compared using Tukey´s test at $p \leq 0.05$ with the aid of SPSS version 20.0. Percentage data were subjected to arcsine transformation before analysis and were converted back to percentages for presentation.

## RESULTS AND DISCUSSION

Rice (*O. sativa*) is a monocot plant species comprising Indica and Japonica subspecies. The *O. sativa* Fujisaka 5 is a cold-resistance japonica rice with high-yielding and fast maturing qualities. *B. decumbens*, a tropical and perennial savanna grass, possess disease and insect resistance, cold and flood tolerance genes with high grain quality (Dai et al. 1990; Low 2015). Both *O. sativa* Fujisaka 5 and *B. decumbens* are good candidates for future rice improvement via genetic transformation and somatic hybridization techniques. Therefore, to use these plant species in the crop improvement program, the prerequisite is the establishment of embryogenic calli and plant regeneration protocol in this japonica rice genotype and *B. decumbens.* The establishment of embryogenic calli using mature seeds and both species' plant regeneration abilities have not been studied and reported elsewhere.

**Establishment of aseptic seed explants**

The aseptic seeds of *O. sativa* Fujisaka 5 (83.3%) and *B. decumbens* (100%) were successfully established by surface-sterilized with 70% ethanol for 10 minutes, followed by 20% Clorox® added with a few drops of Teepol for 30 minutes. After 10 days of culture on MS basal medium, 97.7% of the *O. sativa* Fujisaka 5 seeds germinated while only 40.0% germinated seed were obtained for *B. decumbens* (Table 1). The percentage of seed germinated was significantly different between these two species, but it did indicate the viability of the seeds after the surface sterilization treatment.

**Table 1.** Percentage of established aseptic seeds and germination rate of *Oryza sativa* Fujisaka 5 and *Brachiaria decumbens*

| Plant species | Aseptic seeds (%) | Seed germination (%) |
|---|---|---|
| *O. sativa* Fujisaka 5 | 83.3 [b] | 97.7 [a] |
| *Brachiaria decumbens* | 100 [a] | 40.0 [b] |

Mean values within the same column followed by different letters are significantly different at p≤0.05 (Student t-test)

## Callus induction

Rice calli have been induced from a wide range of explants, such as mature seeds (Khan et al. 2019), immature embryos (Hoo et al. 2021), anther (Ali et al. 2021), and root pieces (Guo et al. 2018), using different types of culture media and PGRs. The aseptic mature seeds of *O. sativa* Fujisaka 5 and *B. decumbens* showed good response in callus production in biomass when cultured on MS medium supplemented with various concentrations (2-10 $mgL^{-1}$) of 2,4-D. After four weeks of culture, the amount of callus formed from the mature seeds of *O. sativa* Fijisaka 5 was not dependent on the amount of 2,4-D added into the MS medium. For *B. decumbens*, the amount of callus produced was not significantly different from that of 2,4-D supplemented into the MS medium. Most of the calli formed were mainly from the mesocotyl and radicles of the germinating embryos (Figure 1.A). Even though the initial induction of callus was only 40 ± 2.2% for *O. sativa* Fujisaka 5 and 25.8 ± 1.2% for *B. decumbens*, the induced callus of the two studied species continued to proliferate well when they were subcultured onto MS medium supplemented 2 $mgL^{-1}$ 2,4-D (Table 2). After five subculture cycles (4 weeks/cycle) using the proliferation medium (MS + 2$mgL^{-1}$ 2,4-D), *O. sativa* Fujisaka 5 was mainly made up of friable and non-embryogenic type (Figure 1.B, 1.C). These non-embryogenic calluses were soft, friable, and pale yellow. While for *B. decumbens*, all the calli formed after five subculture cycles on the same proliferation medium were pale purplish green embryogenic type (Figure 1.D). MS Medium supplemented with 2 $mgL^{-1}$ 2,4-D was selected as the best callus proliferation medium because as the concentration of 2,4-D increased, more browning of the induced calli was observed. Pathania and Sandhu (2021) also reported that an increasing concentration of 2,4-D and an increasing frequency of subculturing resulted in a higher incidence of browning in rice calli. However, until today, 2.4-D is still commonly used for various types of rice cultivars (Sathish et al. 2018; Meesook et al. 2020) but has not been reported for callus induction of *O. sativa* Fijisaka 5 and *B. decumbens*.

## Induction of embryogenic callus of *O. sativa* Fujisaka 5

Since the induced callus of *O. sativa* Fujisaka 5 remained friable and non-embryogenic even after five subculture cycles (4 weeks/cycle) using MS medium supplemented with 2 $mgL^{-1}$ 2,4-D, other plant growth regulators and different MS strengths were employed to study their effectiveness for inducing the formation of embryogenic callus.

**Table 2.** Amount of callus induced from the mature seed explants of *Oryza sativa* Fujisaka 5 and *Brachiaria decumbens* after initial four weeks of culture on MS medium supplemented with 2,4-D

| Plant species | Concentration of 2,4-D ($mgL^{-1}$) | | | | | | Callus type |
|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 10 | |
| | Fresh biomass of callus (mg ± s.e) | | | | | | |
| *O. sativa* Fujisaka 5 | No callus growth | 40 ± 2.2 a | 26 ± 1.6 c | 35 ± 2.5 b | 33 ± 1.2 bc | 36 ± 1.8 b | Friable callus |
| *B. decumbens* | No callus growth | 25.8 ± 1.2 a | 27.1 ± 2.2 a | 24.1 ± 5.0 a | 23.4 ± 6.5 a | 24.5 ± 6.2 a | Embryogenic callus |

Note: Mean values within the same row followed by the same letter are not significantly different at p≤0.05 (Tukey´s honest significance test)

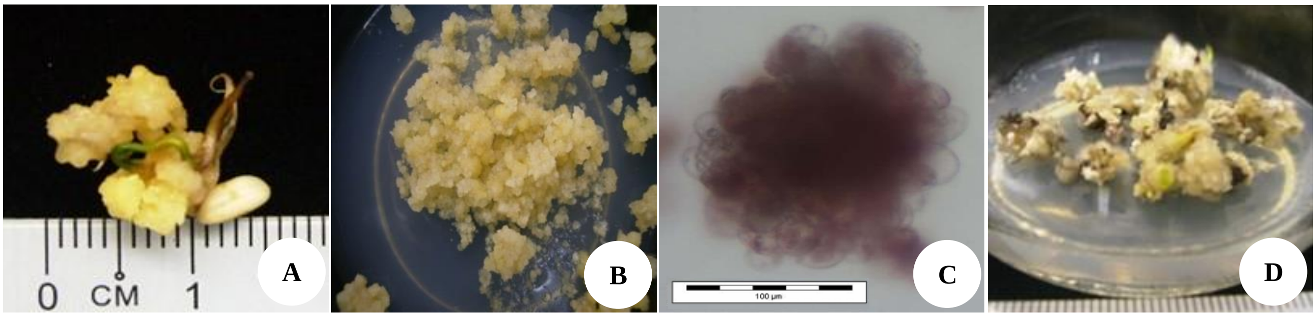


**Figure 1**. A. Formation of callus at the mesocotyl and radicles of the germinating embryos. B. Friable callus clumps of *Oryza sativa* Fujisaka 5. C. Unorganized callus clumps cells of *O. sativa* Fujisaka 5 view under a microscope (40x magnification). D. Pale purplish green embryogenic callus of *Brachiaria decumbens,* after four weeks of culture on MS medium supplemented with 2 $mgL^{-1}$ 2,4-D

*Effect of MS strength*

When the friable callus of *O. sativa* Fujisaka 5, derived from MS supplemented with 2 $mgL^{-1}$ 2,4-D, were cultured onto different strengths of MS-salts without any PGR, more than 80% of the callus formed yellow embryogenic callus and the percentage of embryogenic callus formation was not significantly different among the different MS strength. When the MS-salts decreased, 9.5-19.2% of the callus turned brown and remained friable. Only 3.2% of the calli remained friable when full MS-salts were used. The Growth Index (GI) of the embryogenic callus was also reduced as the MS strength decreased (Table 3). Hence full-strength MS medium was used for the induction of embryogenic callus for the subsequent studies. Several different basal media such as MS (Murashige and Skoog 1962), LS (Linsmaier and Skoog 1965) and N6 (Chu 1978) have been used in rice tissue culture. Vennapusa et al. (2015) have applied these three basal media for callus induction and regeneration for a drought-tolerant rice indica genotype and found that the highest embryogenic callus induction frequency (91.3%) was established using LS basal medium supplemented with 2,4-D. However, until today many researchers still found full MS with a combination of different PGRs are suitable for induction of embryogenic callus in various rice cultivars, such as Himalaya rice (Noor et al. 2022), indica and japonica rice subspecies (Carsono et al. 2021), and Malaysia indica rice (Ng at al. 2019).

*Effect of 2,4-D and kinetin*

After four weeks of culture on MS medium containing 2,4-D or kinetin or combining 2,4-D and kinetin, three colored types of embryogenic calli were formed. The embryogenic calli formed were mostly the yellowish type (85.8-92.6%), and the remainder were of the greenish and brownish type of embryogenic callus. There was no significant difference in the percentage formation of yellowish embryogenic callus type in all tested concentrations 2,4-D and kinetin combination. The combination of 2,4-D and kinetin supplemented into the MS medium greatly affected the formation of the greenish and brownish type of embryogenic callus. The growth of the embryogenic callus based on growth index was high when they were cultured on MS medium without 2,4-D and kinetin, or only with the presence of 2,4-D (0.5$mgL^{-1}$, 1.0 $mgL^{-1}$) or with the presence of both 2,4-D (0.5$mgL^{-1}$, 1.0 $mgL^{-1}$) and kinetin (1.0 $mgL^{-1}$) (Table 4). However, only the green and yellowish type of embryogenic callus could grow if they were subcultured onto the selected embryogenic callus maintenance medium (MS + 0.5 $mgL^{-1}$ 2,4-D + 1.0 $mgL^{-1}$ kinetin) and remained healthy at the embryogenic state. In contrast, the brownish callus died off after four weeks of culture. Paramasivam and Harikrishna (2020) also reported the addition of 2,4-D (2.5 $mgL^{-1}$) and kinetin (0.1 $mgL^{-1}$) into the MS medium induced embryogenic callus in Malaysian wild red rice. In contrast, Paul and Roychoudhury (2019) induced embryogenic calli in indigenous aromatic indica rice varieties using MS medium containing only 2,4-D (3 $mgL^{-1}$). But both authors have not reported the effect of PGRs on browning of the embryogenic calli as found in our present study. Similarly, in our present study, only 2,4-D (2$mgL^{-1}$) supplementation was required to induce the embryogenic callus of *B. decumbens*.

**Table 3**. Effects of different MS strengths without PGRs on induction of embryogenic callus of *Oryza sativa cv. Fujisaka 5*.

| MS strength | Growth Index (GI) (mean ± s.e) | Percentage formation and embryogenic callus Type | |
|---|---|---|---|
| | | Yellowish embryogenic (%) (mean ± s.e) | Brownish friable (%) (mean ± s.e) |
| Full-strength MS | 4.3 ± 1.8 $^{a}$ | 96.8 ± 3.5 $^{a}$ | 3.2 ± 3.5 $^{b}$ |
| Half-strength MS | 3.4 ± 1.2 $^{ab}$ | 84.0 ± 14.1$^{a}$ | 16.0 ± 14.1 $^{a}$ |
| 1/4-strength MS | 3.1 ± 0.8 $^{ab}$ | 80.8 ± 16.9 $^{a}$ | 19.2 ± 16.9 $^{a}$ |
| 1/5-strength MS | 2.4 ± 0.8 $^{b}$ | 90.5 ± 6.9 $^{a}$ | 9.5 ± 6.9 $^{ab}$ |

Note: Mean values within the same column followed by the same letter are not significantly different at p≤0.05 (Tukey´s honest significance test)

**Table 4.** Effects of 2,4-D and kinetin supplemented into MS medium on formation of Embryogenic callus of *Oryza sativa* cv. Fujisaka 5

| MS medium supplemented with | | Growth Index | Type of callus (% mean ± se) | | |
|---|---|---|---|---|---|
| 2,4-D ($mgL^{-1}$) | Kinetin ($mgL^{-1}$) | (GI) ± se | Greenish | Yellowish | Brownish |
| 0 | 0 | 3.5 ± 0.4$^{a}$ | 2.4 ± 0.2$^{b}$ | 85.8 ± 5.9$^{a}$ | 11.7 ± 2.5$^{a}$ |
| 0.5 | 0 | 3.3 ± 0.5$^{a}$ | 1.8 ± 0.4$^{c}$ | 91.6 ± 2.9$^{a}$ | 6.6 ± 1.0$^{ab}$ |
| 1.0 | 0 | 3.2 ± 0.6$^{a}$ | 0.4 ± 0.4$^{d}$ | 95.6 ± 2.1$^{a}$ | 4.0 ± 0.8$^{c}$ |
| 0 | 0.5 | 1.0 ± 0.3$^{c}$ | 0.8 ± 0.6$^{d}$ | 93.8 ± 2.6$^{a}$ | 5.4 ± 1.2$^{b}$ |
| 0.5 | 0.5 | 2.2 ± 0.6$^{b}$ | 2.3 ± 0.4$^{bc}$ | 89.1 ± 3.1$^{a}$ | 8.7 ± 1.6$^{a}$ |
| 1.0 | 0.5 | 2.1 ± 0.6$^{b}$ | 4.5 ± 0.9$^{a}$ | 93.2 ± 2.1$^{a}$ | 2.3 ± 0.9$^{d}$ |
| 0 | 1.0 | 1.8 ± 0.5$^{b}$ | 1.4 ± 0.2$^{cd}$ | 96.2 ± 1.2$^{a}$ | 2.4 ± 0.8$^{d}$ |
| 0.5 | 1.0 | 3.1 ± 0.6$^{ab}$ | 4.3 ± 0.9$^{a}$ | 91.5 ± 3.3$^{a}$ | 4.2 ± 1.7$^{c}$ |
| 1.0 | 1.0 | 2.7 ± 0.6$^{ab}$ | 0.2 ± 0.2$^{d}$ | 92.6 ± 4.5$^{a}$ | 7.2 ± 1.5$^{a}$ |

Note: Mean values within the same column followed by the same letter are not significantly different at p≤0.05 (Tukey´s honest significance test)

**Table 5.** Effects of NAA and BAP supplemented into MS medium on *Oryza sativa* cv Fujisaka 5 embryogenic callus formation

| MS Medium supplemented with | | Growth Index | Type of callus (% mean ± se) | | |
|---|---|---|---|---|---|
| BAP ($mgL^{-1}$) | NAA ($mgL^{-1}$) | (GI) ± se | Greenish | Yellowish | Brownish |
| 0 | 0 | $5.2 \pm 0.4^{a}$ | $0^{a}$ | $100.0 \pm 0.0^{a}$ | $0^{a}$ |
| 0.5 | 0 | $3.9 \pm 0.4^{ab}$ | $0.1 \pm 0.1^{a}$ | $97.2 \pm 1.5^{a}$ | $2.7 \pm 1.5^{a}$ |
| 1.0 | 0 | $3.9 \pm 0.4^{ab}$ | $0.7 \pm 0.5^{a}$ | $98.2 \pm 0.7^{a}$ | $1.1 \pm 0.7^{a}$ |
| 0 | 0.5 | $4.8 \pm 0.5^{ab}$ | $0^{a}$ | $99.2 \pm 0.4^{a}$ | $0.8 \pm 0.4^{a}$ |
| 0.5 | 0.5 | $3.4 \pm 0.2^{b}$ | $0^{a}$ | $98.3 \pm 1.0^{a}$ | $1.7 \pm 1.0^{a}$ |
| 1.0 | 0.5 | $3.4 \pm 0.3^{b}$ | $0.6 \pm 0.6^{a}$ | $98.6 \pm 0.6^{a}$ | $0.8 \pm 0.3^{a}$ |
| 0 | 1.0 | $2.4 \pm 0.5^{b}$ | $0.8 \pm 0.6^{a}$ | $96.8 \pm 1.7^{a}$ | $2.4 \pm 1.6^{a}$ |
| 0.5 | 1.0 | $3.2 \pm 0.4^{b}$ | $0^{a}$ | $100.0 \pm 0.0^{a}$ | $0^{a}$ |
| 1.0 | 1.0 | $2.7 \pm 0.4^{b}$ | $0.2 \pm 0.2^{a}$ | $99.3 \pm 0.5^{a}$ | $0.4 \pm 0.4^{a}$ |

Note: Mean values within the same column followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey´s honest significance test)

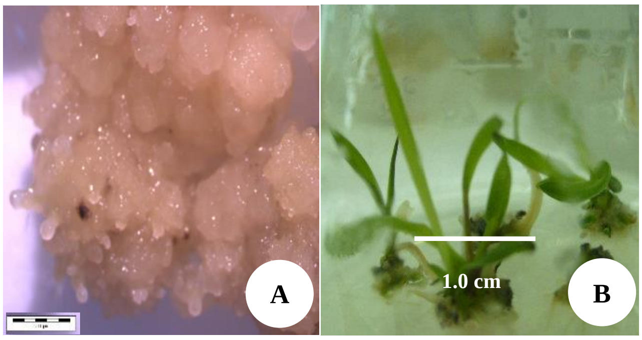


**Figure 2.** A. Somatic embryos of *B. decumbens* cultured on MS medium supplemented with 2 $mgL^{-1}$ 2,4-D. B. Plantlet regeneration of *B. decumbens* after two weeks cultured of embryogenic callus on PGR-free MS medium

*Effect of BAP and NAA*

Most of the friable calli of *O. sativa* Fujisaka 5 formed embryogenic callus (96%-100%) and remained yellowish after four weeks of culture on MS medium without BAP or NAA or MS medium supplemented with 0.5 $mgL^{-1}$ to 1.0 $mgL^{-1}$ BAP and/or NAA. A small amount of the embryogenic callus formed were of the greenish or brownish type. A high growth index was observed in embryogenic callus cultured on MS medium without plant growth regulators or MS medium supplemented with only BAP or NAA (Table 5). Not many researchers reported the effectiveness of BAP and NAA supplemented into MS medium for induction of rice embryogenic callus. However, these two PGRs were efficient for embryogenic callus induction in other plant species such as *Nicotiana tabacum* cv. TAPM26 (Moh Hussein et al. 2020) and *Nigella damascene* L. (Klimek-Chodacka et al. 2020).

**Plant regeneration ability**

*Brachiaria decumbens*

The plant regeneration of *B. decumbens* was straight forward because all the embryogenic callus with greenish somatic embryos derived from MS + 2.0 $mgL^{-1}$ 2,4-D germinated into plantlet after two weeks of transferring onto PGR-free MS medium (Figure 2.A-2.B). An average of 4 to 5 plantlets could be obtained from 0.5g of the inoculated embryogenic callus.

*Oryza sativa cv. Fujisaka 5*

The embryogenic callus of *O. sativa* Fujisaka 5 induced using different MS-salts strength or MS medium without any PGRs could not regenerate into plantlets. After three weeks of culture, the embryogenic callus derived from the lower MS Strength turned brown from the original yellow callus. This might be due to the stress caused by the initial culture in a PGR-free medium and low salt concentration. The lowest MS-strength (1/5 MS-strength) caused the highest percentage of brown callus (up to 90%). The embryogenic callus inoculated on full MS-strength medium exhibited 2% of green embryos but failed to regenerate into plantlets. These results indicated that the embryogenic callus of *O. sativa* Fujisaka 5 derived from any strength of MS medium without any PGRs could not develop into plantlets when transferred directly onto PGR-free MS medium.

Three colored types of embryogenic callus, yellow, green, and brown, were induced when cultured on MS medium supplemented with a combination of 2,4-D and kinetin or MS medium supplemented with a combination of BAP and NAA (Figure 3.A). A high percentage of the embryogenic callus formed was the yellowish type that could not germinate to form plantlets when transferred onto a PGR-free MS medium. Only the greenish type of embryogenic callus, mostly at the heart or torpedo stage of embryos (Figure 3.B), were generated into plantlets after two weeks cultured on the PGR-free MS medium. Via observation, it was found that the plant regeneration ability corresponded well with the amount of greenish type of embryogenic callus formation. These green somatic embryos gave rise to normal plantlets when sub-cultured onto fresh PGR-free MS medium after two weeks (Figure 3.C). MS medium + 1.0 $mgL^{-1}$ 2,4-D + 0.5 $mgL^{-1}$ kinetin or MS medium + 0.5 $mgL^{-1}$ 2,4-D + 1.0 $mgL^{-1}$ kinetin could induce the formation of 4.3 to 4.5% of green embryogenic callus (Table 4) and these green embryogenic calli continue to proliferate well when they were subcultured on these proliferative media. Continuous subculturing on these proliferative media produced the green embryogenic calli which could be used to regenerate plantlets. An average of two to three plantlets could be developed from 0.5 g of the inoculated embryogenic callus after two weeks transferred onto a PGR-free MS medium.

This present study showed that the low regeneration ability of *O. sativa* Fujisaka 5 was largely due to the problem of the low germination rate of the induced somatic embryos. Several factors, including plant genotypes, explant type, plant growth regulators, and culture conditions, could subsequently affect somatic embryos germination and plant regeneration (Ghobeishavi et al. 2015). Cellular differentiation and accumulation of reserve substances are also key factors for successfully converting somatic embryos into plantlets (Mishra et al. 2012). Mature seed explants were found to have a low frequency of plant regeneration from somatic embryos to plant regeneration (Verma et al. 2011).

**Histology of *Oryza sativa* cv. Fujisaka 5 somatic embryos**

Histological study of *O. sativa* Fujisaka 5 somatic embryos derived from the induced embryogenic callus (Figure 4.A) showed primordial leaves and meristematic shoot formation with plant regeneration after two weeks cultured on the PGR-free MS medium. The differentiated primordial leaves and shoot comprised small isodiametric cells with prominent nuclei and dense cytoplasm (Figure 4.B). After four weeks of culture, a complete plantlet was formed with well-developed roots.

Results of our study showed that *in vitro* plant regeneration of *B. decumbens* and *O. sativa* Fujisaka 5 could be established via somatic embryogenesis technique but with a slightly different protocol. Plant regeneration of *B. decumbens* could be accomplished via direct somatic embryogenesis, which involved two stages, initiation of somatic embryos on MS medium supplemented with 2 mgL$^{-1}$ 2,4-D and plantlet regeneration after transferring the somatic embryos onto PGR-free MS medium. For *O. sativa* Fujisaka 5, plantlet regeneration was established via indirect somatic embryogenesis process. It involved three stages, formation of non-embryogenic callus on MS medium supplemented with 2 mgL$^{-1}$ 2,4-D, followed by induction of embryogenic callus and somatic embryos using MS medium + 1.0 mg/L 2,4-D + 0.5 mg/L kinetin or MS medium + 0.5 mg/L 2,4-D + 1.0 mg/L kinetin. Finally, plant regeneration could be achieved by transferring these embryogenic calli and somatic embryos onto a PGR-free MS medium.

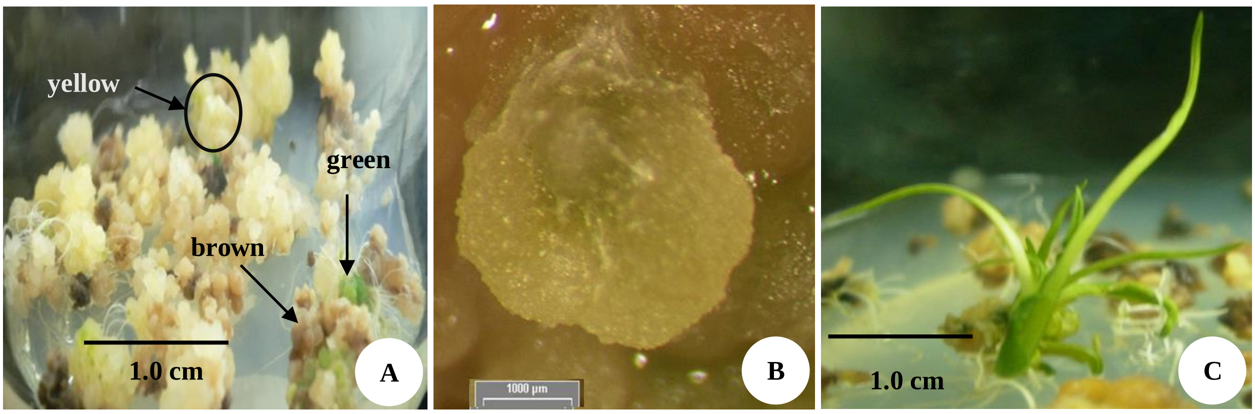


**Figure 3.** A. Mixture of yellow, green and brown embryogenic callus with some friable callus and somatic embryos of *Oryza sativa* Fujisaka 5 established on MS medium + 1.0 mgL$^{-1}$ 2,4-D + 0.5 mgL$^{-1}$ kinetin. B. Heart/torpedo stage of somatic embryos existing in green embryogenic callus of culture (A). C. Plantlet regeneration of *O. sativa* Fujisaka 5 after two weeks cultured of (A) on PGR-free MS medium.

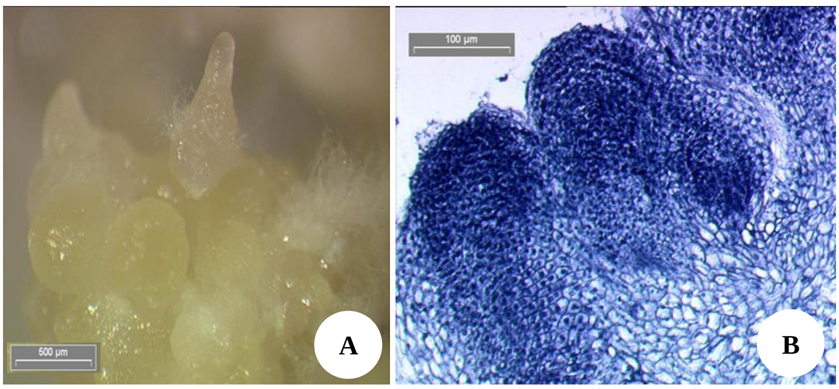


**Figure 4.** A. Somatic embryos of globular and Torpedo stage developed from the induced embryogenic callus. B. Formation of primordial leaves and meristematic shoot of germinated *Oryza sativa* Fujisaka 5 somatic embryo

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