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Best plant harmon combination for *In vitro* callus initiation, organogenesis and regeneration of rice *cv*. swarna sub1

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Abstract
The rapid increase of population is not in tally with the increase of crop production which is leading the world to a severe food crisis. It has become the need-of-the-hour to increase the production of cereals by introducing traits like high yield, disease tolerance, submergence tolerance, etc. Mature de husked rice seed most suitably sterilized with $HgCl_2$ (0.1%) treatment for 5 minutes and observed that media containing 2, 4-D (1.5 mg/l) + BAP (0.5 mg/l) supported maximum callus induction and a percentage of 80% was obtained which was highly organogenic also. When the Somatic embryo of Swarna Sub1 inoculated in MS media supplemented with NAA (0.5 mg/l) +BAP (3 mg/l) + KIN (0.5 mg/l) produces average 10 shoot and 4 root initiation. The protocols developed will facilitate large scale propagation of the transformed plants.

Keywords: swarna sub1, callus induction, shoot induction, root induction, seed sterilization

Introduction
The introduction of beneficial genes from other organisms /plants such as those encoding disease and insect resistance *via* into the rice genome arose from developments in this area. Considerable improvement made through tissue culture and genetic transformation techniques are being employed in rice development for the creation of novel rice varieties. However, the production of embryogenic calli and its subsequent regeneration are the basic prerequisites for the potential use of molecular techniques. Successful embryogenic calli induction is influenced and depended on many factors such as plant genotype, explant type, culture medium, plant growth regulators and culture environment. The induction of high-quality rice callus influenced by genotype, medium, and the kind of explants as well as their interactions. Callus induction and regeneration is still a challenging task in most rice varieties (Shukla *et al.*, 2014) [15]. The purpose of this study was to develop a reproducible and an efficient procedure for callus induction and plant regeneration of embryogenic calli from mature seeds of Swarna *Sub1* for future genetic transformation studies.

Material and methods
The work was conducted in Plant Tissue Culture Laboratory, Jacob School of Biotechnology and Bioengineering, Sam Higginbottom Institute of Agriculture, Technology and Sciences, Allahabad. The rice de- husked mature rice seeds were washed for 20 minutes in running tap water for removal of dust followed by disinfecting with laboratory detergent (Domex, 15%) solution for 15 to 20 minutes. Again rinsed in double distilled water and transferred to the laminar hood. The explants were then disinfected with several surfactants for several time and concentrations and finally rinsed with sterilized distilled water seven times in laminar air flow cabinet and decanted on the sterile filter paper surface. Rice seeds are inoculated on MS media (Murashige and Skoog, 1962) [9] at different concentrations for callus induction, embryogenesis and organogenesis purpose.

Results and Discussion
Seed Sterilization
Different concentration of surfactants for several times were used for de husked mature rice seed sterilization purpose were selected and inoculated under aseptic condition on a week old autoclaved MS media. After thirty six days of rice seed inoculation, only two treatments of $HgCl_2$ 0.1% for three minutes and five minutes were found more effective (Islam *et al.*, 2004; Roy and Mandal, 2005; Agasian *et al.*, 2006) [5, 12, 1], than compare to other treatment (Table1).

Table 1: Sterilizing agents and their efficiency on *in vitro* culture of rice seeds express in contamination percentage

Days	$HgCl_2$ 0.01%			$HgCl_2$ 0.1%			C_2H_5OH 70%			NaClO 15%						H_2O_2 10%			H_2O_2 15%		
	1min	3min	5min	1min	3min	5min	5min	10min	15min	1min	3min	5min	10min	15min	20min	1min	3min	5min	5min	10min	15min
00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00
03	00.00	00.00	00.00	00.00	00.00	00.00	33.33	33.33	00.00	33.33	66.67	33.33	00.00	00.00	33.33	33.33	33.33	66.67	00.00	00.00	00.00
06	00.00	00.00	00.00	00.00	00.00	00.00	100	66.67	66.67	33.33	33.33	66.67	66.67	33.33	33.33	100	66.67	100	66.67	33.33	0.00
09	33.33	66.67	33.33	00.00	00.00	00.00	100	66.67	100	66.67	66.67	66.67	100	66.67	100	100	66.67	100	66.67	66.67	33.33
12	66.67	66.67	33.33	00.00	00.00	00.00	100	100	100	100	100	100	100	66.67	100	100	100	100	66.67	66.67	33.33
15	66.67	66.67	33.33	00.00	00.00	00.00	100	100	100	100	100	100	100	66.67	100	100	100	100	66.67	66.67	33.33
18	66.67	66.67	33.33	33.33	00.00	00.00	100	100	100	100	100	100	100	66.67	100	100	100	100	66.67	66.67	33.33
21	66.67	66.67	33.33	33.33	00.00	00.00	100	100	100	100	100	100	100	66.67	100	100	100	100	66.67	66.67	33.33
24	66.67	66.67	33.33	33.33	00.00	00.00	100	100	100	100	100	100	100	66.67	100	100	100	100	66.67	66.67	33.33
27	66.67	66.67	33.33	66.67	00.00	00.00	100	100	100	100	100	100	100	66.67	100	100	100	100	66.67	66.67	33.33
30	66.67	99.67	33.33	100	00.00	00.00	100	100	100	100	100	100	100	66.67	100	100	100	100	66.67	66.67	33.33
33	66.67	100	33.33	100	00.00	00.00	100	100	100	100	100	100	100	66.67	100	100	100	100	66.67	66.67	33.33
36	66.67	100	33.33	100	00.00	00.00	100	100	100	100	100	100	100	66.67	100	100	100	100	66.67	66.67	33.33

Callus induction

Swarna *Sub1* seed embryo were treated with fifty six different hormone combinations and the data was recorded for rate of callus formation and three different phenotypic characters, viz. callus appearance, callus colour and callus type (Table 2). The callus induction percentage was highest in SS29 (80.00%) followed by SS4 which gave 76.67%, SS33 and SS44 gave 66.67% and SS5, SS18, SS38 and SS56 gave 63.33% (Table 2; Figure a). The auxins, 2,4-D is a potent growth substance which is metabolized by tissues more slowly and hence retained longer in medium than NAA and is more active in triggering the meristematic activity leading to cell proliferation (Wagiran *et al.,* 2008; Kazzaz *et al.,* 2009, Sharma *et al.,* 2017) [19, 6, 17].

Htwe *et al.* (2011) [4] and Sharma *et al.* (2017) [14] isolated the embryogenic callus and selected them based on the description by Van *et al.* (1990) [18] which is described as usually light yellow to creamy white in colour, as well as dry, compact and nodular. Results obtained by Rashid *et al.* (2009) [11] that increase in the concentration of 2, 4-D and kinetin reduced the percentage of embryogenic calli production. Moreover, difference in callus proliferation rate between different auxins may be due to the difference in the physiological activity of the auxins (Wagiran *et al.,* 2008) [20] and differences in response of genotypes, especially even when carried out after a short time of callus maintenance (Muhammad *et al.,* 2005; Htwe *et al.,* 2011) [8, 4]. Embryogenic calli were relatively smooth (oily), compact, nodular in appearance and milky white (yellowish) in colour (Rachmawati and Anzai, 2006; Mahmood *et al.,* 2012, Shukla *et al*, 2014, Sharma *et al.,* 2017) [10, 7, 15, 14].

Table 2: Effect of different plant hormones on rice callus induction, appearance, colour and type

Sl. No	Media Code	Hormonal Concentration mg/l	Callus Induction %	Callus formation rate	Callus Appearance	Callus Colour	Callus Type
1.	SS1	M.S. Basal	0.00 ± 0.00^{k}	-	-	-	-
2.	SS2	0.5mg/l 2,4D	43.33 ± 4.71^{efg}	++	Dry	Light Yellow	Compact
3.	SS3	1.0 mg/l 2,4D	56.67 ± 4.71^{cde}	+++	Oily	Brownish Yellow	Compact
4.	SS4	1.5 mg/l 2,4D	76.67 ± 4.71^{ab}	++++	Oily	Light Yellow	Compact
5.	SS5	2.0 mg/l 2,4D	63.33 ± 4.71^{bcd}	++++	Oily	Light Yellow	Compact
6.	SS6	2.5mg/l 2,4D	56.67 ± 4.71^{cde}	+++	Oily	Light Yellow	Compact
7.	SS7	3.0 mg/l 2,4D	56.67 ± 4.71^{cde}	+++	Oily	Dark Yellow	Compact
8.	SS8	3.5 mg/l 2,4D	46.67 ± 4.71^{ef}	++	Dry	Yellow	Compact
9.	SS9	4.0 mg/l 2,4D	43.33 ± 4.71^{efg}	++	Dry	Yellow	Compact
10.	SS10	4.5 mg/l 2,4D	43.33 ± 4.71^{efg}	++	Oily	Blackish Brown	Compact
11.	SS11	5.0 mg/l 2,4D	30.00 ± 8.16^{ghi}	+	Oily	Black	Compact
12.	SS12	0.5 mg/l 2,4-D +0.5mg/l NAA	16.67 ± 9.47^{ij}	+	Dry	Light Yellow	Compact
13.	SS13	1.0 mg/l 2,4-D +0.5mg/l NAA	26.67 ± 4.71^{hij}	+	Oily	Brownish Yellow	Compact
14.	SS14	1.5 mg/l 2,4-D +0.5mg/l NAA	30.00 ± 8.16^{ghi}	+	Dry	Brownish Yellow	Friable
15.	SS15	2.0 mg/l 2,4-D +0.5mg/l NAA	30.00 ± 8.16^{ghi}	+	Dry	Brownish Yellow	Friable
16.	SS16	2.5 mg/l 2,4-D +0.5mg/l NAA	26.67 ± 4.71^{hij}	+	Dry	Black	Compact
17.	SS17	0.5 mg/l 2,4-D +1.0mg/l NAA	43.33 ± 4.71^{efg}	++	Dry	Brownish Yellow	Compact
18.	SS18	1.0 mg/l 2,4-D+ 1.0mg/l NAA	63.33 ± 4.71^{bcd}	++++	Oily	Light Yellow	Compact
19.	SS19	1.5 mg/l 2,4-D + 1.0mg/l NAA	46.67 ± 4.71^{ef}	+++	Oily	Light Yellow	Compact
20.	SS20	2.0 mg/l 2,4-D + 1.0mg/l NAA	13.33 ± 9.43^{jk}	+	Oily	Brownish Yellow	Friable
21.	SS21	2.5 mg/l 2,4-D + 1.0mg/l NAA	56.67 ± 4.71^{cde}	+++	Oily	Light Yellow	Compact
22.	SS22	0.5mg/l 2,4-D+ 1.5mg/l NAA	30.00 ± 8.16^{ghi}	+	Oily	Deadly Brown	Compact
23.	SS23	1.0 mg/l 2,4-D + 1.5mg/l NAA	30.00 ± 8.16^{ghi}	+	Oily	Brownish Yellow	Compact
24.	SS24	1.5 mg/l 2,4-D +1.5mg/l NAA	26.67 ± 4.71^{hij}	+	Dry	Light Brown	Friable
25.	SS25	2.0 mg/l 2,4-D+ 1.5mg/l NAA	16.67 ± 9.47^{ij}	+	Dry	Brownish Yellow	Friable
26.	SS26	2.5 mg/l 2,4-D + 1.5mg/l NAA	16.67 ± 9.47^{ij}	+	Dry	Brownish Yellow	Friable
27.	SS27	0.5 mg/l 2,4-D+ 0.5mg/l BAP	43.33 ± 4.71^{efg}	++	Oily	Brownish Yellow	Compact
28.	SS28	1.0 mg/l 2,4-D +0.5mg/l BAP	50.00 ± 8.16^{def}	++	Oily	Brownish Yellow	Friable
29.	SS29	1.5 mg/l 2,4-D+ 0.5mg/l BAP	80.00 ± 0.00^{a}	++++	Oily	Light Yellow	Compact
30.	SS30	2.0 mg/l 2,4-D+0.5mg/l BAP	56.67 ± 4.71^{cde}	+++	Oily	Brownish Yellow	Friable

31.	SS31	2.5 mg/l 2,4-D, 0.5mg/l BAP	53.33 ±4.71^{cde}	+++	Oily	Deadly Brown	Friable
32.	SS32	0.5 mg/l 2,4-D + 1.0mg/l BAP	50.00 ±8.16^{def}	++	Dry	Dark Yellow	Friable
33.	SS33	1.0 mg/l 2,4-D+ 1.0mg/l BAP	66.67 ±4.71^{abc}	++++	Oily	Light Yellow	Compact
34.	SS34	1.5 mg/l 2,4-D+ 1.0mg/l BAP	36.67 ±8.47^{fgh}	++	Oily	Deadly Brown	Friable
35.	SS35	2.0 mg/l 2,4-D+ 1.0mg/l BAP	26.67 ±4.71^{hij}	+	Oily	Brownish Yellow	Friable
36.	SS36	2.5 mg/l 2,4-D+ 1.0mg/l BAP	26.67 ±4.71^{hij}	+	Oily	Brownish Yellow	Friable
37.	SS37	0.5 mg/l 2,4-D+ 1.5mg/l BAP	43.33 ±4.71^{efg}	++	Oily	Dark Yellow	Compact
38.	SS38	1.0 mg/l 2,4-D+ 1.5mg/l BAP	63.33 ±4.71^{bcd}	++++	Oily	Light Yellow	Compact
39.	SS39	1.5 mg/l 2,4-D+ 1.5mg/l BAP	46.67 ±4.71^{ef}	++	Dry	Deadly Brown	Compact
40.	SS40	2.0 mg/l 2,4-D+ 1.5mg/l BAP	43.33 ±4.71^{efg}	++	Dry	Light Yellow	Friable
41.	SS41	2.5 mg/l 2,4-D+ 1.5mg/l BAP	43.33 ±4.71^{efg}	++	Dry	Light Yellow	Friable
42.	SS42	0.5 mg/l 2,4-D+ 0.5mg/l Kin	53.33 ±9.47^{cde}	+++	Oily	Light Yellow	Compact
43.	SS43	1.0 mg/l 2,4-D+ 0.5mg/l Kin	56.67 ±4.71^{cde}	+++	Dry	Dark Yellow	Friable
44.	SS44	1.5 mg/l 2,4-D+ 0.5mg/l Kin	66.67 ±9.43^{abc}	++++	Oily	Light Yellow	Compact
45.	SS45	2.0 mg/l 2,4-D+ 0.5mg/l Kin	53.33 ±4.71^{cde}	+++	Oily	Brownish Yellow	Compact
46.	SS46	2.5 mg/l 2,4-D+ 0.5mg/l Kin	43.33 ±4.71^{efg}	++	Dry	Brownish Yellow	Friable
47.	SS47	0.5 mg/l 2,4-D+ 1.0mg/l Kin	13.33 ±9.43^{jk}	+	Oily	Light Brown	Compact
48.	SS48	1.0 mg/l 2,4-D+ 1.0mg/l Kin	16.67 ±9.47^{ij}	+	Dry	Light Yellow	Friable
49.	SS49	1.5 mg/l 2,4-D+ 1.0mg/l Kin	36.67 ±4.71^{fgh}	++	Oily	Brownish Yellow	Compact
50.	SS50	2.0 mg/l 2,4-D+ 1.0mg/l Kin	56.00 ±4.71^{cde}	+++	Oily	Deadly Brown	Compact
51.	SS51	2.5 mg/l 2,4-D+ 1.0mg/l Kin	26.67 ±9.43^{hij}	+	Oily	Brownish Yellow	Compact
52.	SS52	0.5 mg/l 2,4-D+ 1.5mg/l Kin	23.33 ±4.71^{hij}	+	Oily	Brownish Yellow	Compact
53.	SS53	1.0 mg/l 2,4-D+ 1.5mg/l Kin	46.67 ±4.71^{ef}	+	Oily	Deadly Brown	Compact
54.	SS54	1.5 mg/l 2,4-D+ 1.5mg/l Kin	56.00 ±4.71^{cde}	+++	Oily	Brownish Yellow	Compact
55.	SS55	2.0 mg/l 2,4-D+ 1.5mg/l Kin	50.00 ±8.16^{def}	++	Oily	Brownish Yellow	Compact
56.	SS56	2.5 mg/l 2,4-D+ 1.5mg/l Kin	63.33 ±4.71^{bcd}	++++	Oily	Light Yellow	Compact

- = no callusing; + = meager callusing; ++ = moderate callusing; +++ = high callusing; ++++ = profuse callusing

Values are means of 3 replicates. Mean values followed by the same letters are not significantly different at p ≥ 0.05 DMRT

***In vitro* plant regeneration**

Itcan be inferred that an auxin: cytokinin combination is favorable for shoot initiation than addition of cytokinin alone in the media. The two cytokinins, BAP and KIN were required along with the auxin NAA to support maximum shoot induction, with a higher BAP concentration than KIN. When the cytokinins BAP and KIN were supplemented in the media without the auxin, the number of shoots developed was lesser than the combination (Saha *et al*., 2017) [13]. Also, when the auxin was supplemented along with only one cytokinin, it supported only less shoot initiation. The media containing NAA 0.5 mg/l +BAP 3 mg/l + KIN 0.5 mg/l (RM 12; Figure b,c &d) gave the maximum number of shoots and roots of 10 and 4 respectively (Table 3) and thus was selected for further studies (Figure e) on plantlet regeneration of calli obtained from the earlier selected media. Usually, shoot bud induction from callus is the function of cytokinin activity and relatively high ratio of cytokinin to auxin is mandatory for shoot initiation from calluses (Subbaiah and Minocha, 1990, Aygunand Dumanoglu, 2015; Saha *et al*., 2017; Bekircan 2018; Stevens and Pijut, 2018) [17, 2, 13, 3, 16].

Table 3: Mean for regeneration performance of selected callus

Media Code	Hormone concentration in media (mg/l)			No. of Shoots/ Callus	No. of Roots/ Callus
	NAA	BAP	KIN		
RM 1	0.0	0.0	0.0	1.0±00^{e}	02±0.7^{b}
RM 2	0.0	1.5	0.5	07±2.4^{bc}	02±0.2^{bc}
RM 3	0.0	1.5	1.0	06±4.2^{c}	01±0.0^{c}
RM 4	0.0	1.5	1.5	05±2.3^{cd}	03±0.5^{ab}
RM 5	0.5	1.5	0.0	08±1.2^{b}	04±0.4^{a}
RM 6	1.0	1.5	0.0	07±3.2^{bc}	03±0.2^{ab}
RM 7	1.5	1.5	0.0	05±3.6^{cd}	01±0.0^{c}
RM 8	0.5	0.0	0.5	06±4.2^{c}	00±0.0^{d}
RM 9	1.0	0.0	0.5	06±4.2^{c}	01±0.0^{c}
RM 10	1.5	0.0	0.5	06±3.5^{c}	02±0.3^{bc}
RM 11	0.5	1.5	0.5	05±2.3^{cd}	01±0.1^{bc}
RM 12	0.5	3.0	0.5	10±0.2^{a}	04±0.4^{a}
RM 13	0.5	5.0	0.5	03±1.1^{d}	01±0.1^{bc}

Values are means of 3 replicates. Mean values followed by the same letters are not significantly different at p ≥ 0.05 DMRT

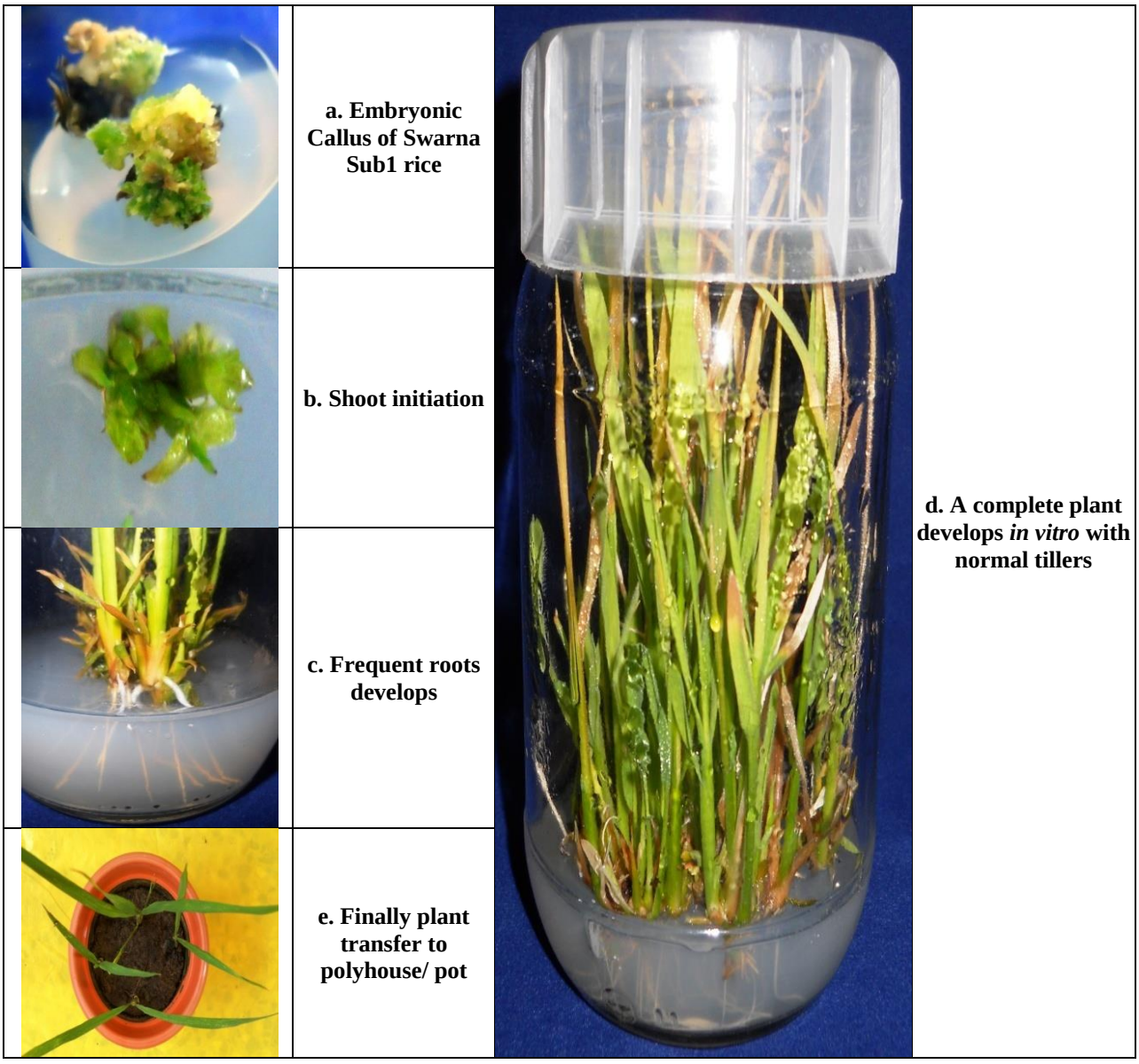

Fig 1: Representing the different *in vitro* developmental stages of rice

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