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Physio-Molecular approach towards developing rice variety for dual resistance (Drought and Submergence) under rainfed lowland ecosystem

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Abstract
Crop in Rainfed lowland ecosystem often suffers both drought and/or submergence (either initial or last phase of life cycle) sometime in same life cycle depending upon prevalent weather in their region. Rice is more prone to this leading very poor or no yield. In the present study, one of promising IRRI rice lines NDR 9830102 was evaluated for drought and submergence. Swarna sub 1 and Jalmagna was taken as positive control for submergence and Nagina 22 as positive control for drought. Swarna and Mahsuri were also evaluated in both conditions. For drought observation were recorded on leaf rolling, relative water content and proline content while for submergence observation were recorded plant height, shoot elongation, survival% and carbohydrate content. Experimental results after desubmergence revealed that the NDR 9830102, Jalmagna had maximum shoot elongation followed by Swarna, Mahsuri while Swarna sub 1 had minimum shoot elongation. Swarna, Mahsuri, NDR 9830102 had minimum survival% and carbohydrate accumulation while Swarna sub 1 and Jalmagna had maximum survival%, carbohydrate accumulation. Under drought condition accumulation of proline were recorded maximum in Nagina 22 followed by NDR 9830102, Swarna and Swarna sub 1. Genetic diversity analysis by SDS-PAGE reveals that the genotype Swarna, Swarna sub 1 and NDR 98102 showed remarkable variation among each other. Seeing the promising dual tolerance in NDR 9830102, crosses were made between Swarna sub1 x NDR 9830102 and their F_1 was tested through RAPD marker (OPA 11).

Keywords: Rainfed lowland, shoot elongation, carbohydrate, proline, protein and DNA

Introduction
Global climate change influences the frequency and magnitude of hydrological fluctuations, causing devastating events such as floods and drought. Both high and low extremes in precipitation increasingly limit food, fiber, and forest production worldwide (Easterling *et al*., 2007) [8]. Approximately 30% of the world's rice (*Oryza sativa*) farmlands are at a low elevation and irrigated by rain (Bailey-Serres *et al*., 2010) [2]. However, rain-fed fields are prone to flooding and drought due to inadequate water management. Thus, submergence, drought, and the sequential events (submergence followed by drought and vice versa) are major constraints to rice production in rainfed lowlands. Therefore, improvement of combined tolerance to submergence and drought would substantially increase rice productivity while sustaining water resources and soil quality.

Rice is a semi aquatic species that is typically cultivated under partially flooded conditions. However, flash flooding can cover the entire plant for prolonged periods, and most rice cultivars die within 7 d of complete submergence (Xu *et al*., 2006; Bailey-Serres *et al*., 2010) [46, 2]. A limited number of rice cultivars overcome submergence through antithetical growth responses. Deepwater rice responds to submergence by promoting internode elongation to outgrow floodwaters. By contrast, submergence tolerant lowland rice, including Flood Resistant 13A (FR13A), restrains elongation growth, economizing carbohydrate reserves to enable development of new leaves upon desubmergence. This quiescence response is regulated by another AP2/ERF, located at the polygenic SUBMERGENCE1 (SUB1) locus (Xu *et al*., 2006) [46].

Drought is the most important factor limiting rice productivity in the rainfed rice agro-ecosystem (Huke and Huke, 1997) [15]. Climate change is Predicted to increase the frequency and severity of drought, which will likely result in increasingly serious constraints to rice production worldwide (Wassmann *et al*., 2009) [45]. Drought can occur at any stage of the rice crop in any year in rainfed areas. Modern rice varieties are highly sensitive to drought stress at seedling, vegetative, and reproductive stages and even mild drought stress can result in a significant yield reduction in rice (O'Toole, 1982; Torres and Henry, 2016) [25, 41].

At seedling stage, drought affects crop establishment and seedling survival rates. At vegetative stage, drought reduces leaf formation and tillering, which subsequently reduces the development of panicles per plant, thus causing a yield loss; whereas, at reproductive stage, drought causes a reduction in the number of grains per panicle, increases grain sterility, and reduces grain weight (Pantuwan *et al*., 2002) [30]. Drought causes changes in the physio-biochemical level in plants and thus accumulation of compatible solute as a osmoprotectant, such as proline, glycine, betaine and sugars to impart drought confer resistance in crop.

In the present study, a set of genotypes previously reported to be tolerant of drought at the reproductive stage (Verulkar et. al., 2010; Raman *et al*., 2012) [34] and some additional genotypes identified to be tolerant to reproductive stage drought were evaluated for tolerance of vegetative-stage drought stress. The genotypes were developed by crossing high-yielding but drought-susceptible rice varieties with drought-tolerant donors and subjected to direct selection for grain yield under reproductive-stage drought and well-watered conditions. The genotypes were evaluated under different levels of vegetative-stage drought stress and submergence stress with the aim of (i) characterizing the yield and yield component under moderate and severe vegetative-stage drought stress and submergence stress, and (ii) identifying genotypes that show tolerance of both vegetative- and reproductive-stage drought stress and submergence stress, in addition to having high yield potential under well-watered conditions. (iii) crosses were made among drought tolerant and susceptible high yielding genotypes and their F_{1s} validated by RAPD marker.

Method and material

Submergence treatment

Germinated seeds were transplanted into 2.5-liter soil-containing pots and grown for 45 d. The crop was maintained properly at 80:40; 40 kg/ha NPK level. Submergence stress was applied by withholding water for 12 d in a pond and recovery was initiated by placing pots in a green house without giving water. Plant viability was evaluated 10 d after initiation of recovery under normal growth conditions. Plants survival scored if one or more new leaves appeared during the recovery period.

Drought treatment

The experiments were conducted in the field of NDUAT during 2010 and 2011 rainy seasons. The experimental plots were laid out in random block design with three replications. Rice nursery were prepared in the green house and after 21 days single seedling per hill was transplanted with 20 cm row to row and 15 cm plant to plant spacing in randomized block design (RBD) design with three replication under irrigated and non-irrigated condition. The crop was maintained properly at 120:60:60 kg/ha NPK level. Drought treatment was given at 50 days after sowing.

Proline and total soluble sugar measurement

Free proline was quantified in leaf tissues, according to Bates *et al,* (1973) [3] and total soluble sugar (TSS) was determined by anthrone method given by Yemm, E.W. and Willis, A.J. (1954) [47].

SDS-PAGE analysis of protein

Protein profiles of control, drought-stressed and drought-recovered samples of both the genotypes were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) following the procedure of Laemmli (1970) [22]. A 10% separating gel was prepared and 40 lg of protein solubilized with sample buffer [62.5 mM Tris–HCl, pH 6.8, 20% (w/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue] was loaded in each lane of the gel. Electrophoresis was accomplished at 35 mA for 3 h using Bio-Rad, Protein II electrophoresis system. The gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Sigma) in 50% (v/v) methanol and 10% (v/v) acetic acid for 2 h and destained with 50% (v/v) methanol and 10% (v/v) acetic acid until the background was clear. The gels were photographed and scanned using a densitometer (Alpha Imager, USA) and analyzed with Quantity one software from Alpha Imager.

DNA extraction

The DNA extraction from leaves of parents, F_1 using the CTAB method as described by Murray and Thompson, (1980) [24].

RAPD Programme

For RAPD 25 ng of plant DNA, 2mM dNTP mix, 10 p mole of primer, Taq Buffer (1x), 2 mM $MgCl_2$, 1.25 U of Taq DNA Polymerase were taken and made up to 25 micro litre with autoclaved distilled water. The reaction was performed in a thermal cycler (Eppendorf Master Cycler Gradient, Hamburg, Germany). Reaction conditions were as follows: 94 ºC—3 min, 40 cycles of 94 ºC—30 s, 36 ºC—30 s, 72 ºC—1 min and a final extension of 72 ºC for 10 min. 23 random decamer primers obtained from Operon Technologies (Alameda, CA, USA) were used for this study. PCR products were resolved in a horizontal gel electrophoresis system (Classic CSSU1214, Thermo Electron, MA, USA) along with standard 100-bp ladder on a 1.5% agarose, 1x_ TAE buffer. The DNA was stained with Ethidium Bromide (0.5 mg/ml). The gel was visualized under UV radiation and digitally photographed (Alpha Imager, USA).

Result & Discussion

Effect of submergence on rice genotype

Rice genotypes used in this investigation exhibited distinctively variable responses to submergence in terms of visible injury, underwater elongation and plant survival. Genotypes Jalmagna and NDR 9830102 showed greater elongation due to the imposition of submergence, and their leaf tips came out above the water within 12 days of submergence. The other genotypes however remained under water. Plant height did not increase much in Swarna *Sub1* cultivars, resulted significantly lower elongation compared to other genotypes. Swarna also elongate but at certain height under water. Jalmagna, Swarna-Sub1 and NDR 9830102 showed 90, 80 and 70 percent survival after 10 days of submergence treatment. Among the landraces survival percentage was minimum in Swarna (45%).

Greater elongation will deliver benefits by restoring contact with the air above the floodwater, thus improving internal aeration for aerobic respiration and allowing for partly aerial photosynthesis. It has also been suggested that shoot elongation may be associated with costs, as energy and carbohydrates are needed for cell division and elongation (Voesenek *et al.,* 2004; Pierik *et al.,* 2009) [44, 32]. This may ultimately even cause plant death when energy reserves are depleted before reaching the water surface (Das *et al.,* 2005) [7]. 'Swarna' has relatively better elongation ability than 'Swarna'-*Sub1* when submerged, and this is because of the submergence-induced reduction in growth upon induction of *Sub1*, and the consequent halt in shoot growth (Fukao *et al*., 2006, Fukao and Bailey-Serres 2008) [11, 10]. Consequently 'Swarna'-*Sub1* experienced greater inhibition of growth because of both complete submergence and partial water stagnation due to its shorter stature compared with that of other cultivars. The induced reduction in shoot elongation capacity by *Sub1* upon submergence is an adaptive trait that helps reduce carbohydrate consumption during submergence to ensure better survival (Das *et al.*, 2005, Panda *et al*., 2008) [7, 27]. However, deepwater rice varieties can survive, because of rapid stem elongation with rises in water level, sustained by internode elongation by Catling D (1992) [6]. Ordinary paddy rice cultivars do not initiate internode elongation during the vegetative stage; however, deepwater rice cultivars can elongate internodes even in early growth stages and also develop aerenchyma, the snorkel-like conduits in internodes that allow gaseous exchange. This remarkable internode elongation occurs during seasonal flooding, reaching depths up to several meters, and deepwater rice has growth with daily increases in plant height of 20– 25 cm; ultimately, plants can reach a height of 7 m, in contrast to only 1.5 m when grown in dry or shallow water condition Vergara BS *et al.*, (1976) [42]. These characters allow such cultivars to survive deepwater flood, and deepwater rice cultivars are an important crop in flood-prone areas (e.g. Bangladesh, Thailand and Cambodia) Bhuiyan SI *et al.*, *(*2004) [4].

Impact of submergence on total soluble sugar

Reduction of TSS content after 12 days of submergence was 54.50 in SUB1 (non-elongating), 51 in Jalmagna (elongating), 39.50 in NDR 9830102 (elongating) and 37.50 in Swarna (elongating under water). After 20 days of recovery TSS gradually increased in both genotypes. TSS after recovery was recorded highest in Swarna sub 1 and Jalmagna while moderate in NDR 9830102 followed by Swarna less carbohydrate. Total soluble sugar after submergence showed highly significant positive association with survival% (Table 2). The carbohydrate content of plants was found to be significantly and positively associated with re-generation growth (Panda *et al*. 2008a) [26].

Effect of drought on rice genotype

In drought stress condition maximum reduction of plant height, no. of tillers, no. of grain/panicle, spikelet fertility and grain yield were recorded in Swarna sub 1 followed by Swarna, NDR 9830102 and Nagina 22. In irrigated condition maximum no of tillers, no. of panicle bearing tillers, no. of grain/panicle, spikelet fertility and grain yield were recorded in sub 1 followed by Swarna, NDR 9830102 and Nagina 22. The significant decline in plant height of drought-stressed rice cultivars was concomitant with a significant decrease in the grain yield per plant (Table 3). The reduction in plant height of drought-stressed rice might be due to the decline in cell enlargement and cell division. These results are highly matching with those obtained by Hsiao (2000) [14]. Similar finding was also reported by Kumar *et al*., (2009) [21]. Significant decrease in plant height was also observed in rice genotypes grown under drought stress condition. It is also noticeable that, the grain yield decreased significantly in the two drought-stressed cultivars (Table 3).

Imposition of drought stress reduced the number of tillers per plant. Reuben *et al.*, (1990) [35] supported this result and he reported that water deficit at vegetative stage of growth significantly reduced tiller number in both susceptible and tolerant genotypes of rice but the reduction in tiller number was more in susceptible than in tolerant genotypes. Similar results were reported by Pridashti *et al.*, (2004) [33] that reduction in grain yield largely resulted from the increase in sterile panicle and unfilled grain percentage.

In water stress condition, higher value of RWC was recorded in tolerant rice genotypes as compared to susceptible genotypes (Table 3). Relative water content (RWC) is one of the most important parameter to measure water status of the tissue (Barrs and Weatherley, 1962).

Impact of drought on proline

The multiple roles of proline in protecting plants against abiotic stresses are very well documented. As an osmoticum it is used for osmotic adjustment under salt and drought stresses, protects cell structure and acts as a scavenger of damaging free radicals (Nanjo *et al.*, 1999). High proline accumulation under drought condition have been also observed by a number of workers (Blum, 1989; Pandey and Srivastava, 1989; Hou *et al.*, 1990) [5, 29, 13]. It is also evident that tolerant genotypes accumulated more proline content in comparison to susceptible one. As similar finding was observed in our experiment maximum proline content was recorded in Nagina 22 and NDR 9830102 while minimum in Swarna sub 1 and Swarna (graph).

SDS-PAGE analysis of protein

Fig 3. shows protein profiling of rice genotypes by SDS-PAGE at 15% gel. Protein profiling was done to study the genetic diversity among parent. The genotype Swarna sub 1 and Swarna showed significant variation in banding pattern among genotype NDR 102, Jalmagna and Nagina 22 indicated by an arrow and rest of the band is almost similar or common in all genotypes. This variation might be due to the presence of leaves and seed specific proteins (Miffin *et al.*, 1983 and Shewry *et al.*, 1986) [23, 40]. The major band of these proteins appear at low per cent acrylamide while the fractionized and minor bands appeared at high percent acrylamide gel and lower side of the gel (Payne *et al.*, 1987) [31]. Thus genotype Swarna sub 1 was selected as recipient parent and NDR 102 as a donor parent.

Breeding work

The genotypes were selected on the basis of performance under drought and submergence stress condition. The genotype NDR 102 short duration crop showing resistance to both drought and submergence stress while genotype Swarna sub 1 is high yielding submergence tolerance but showing susceptibility to drought stress thus both were selected as parent and crosses were made between them to develop high yielding resistance variety.

Polymorphic study with RAPD marker and F_1 validation

RAPD primers OPA-11 showed high polymorphism between parent was used to validate the trueness of F_1. As shown in fig. OPA-11 amplified product in F_1 lane having three bands similar to Swarna sub 1 and NDR 102 having two bands, confirming the true hybrid nature of F_1. Iqbal (2005) [17] identified the hybrid of (Donald x PD2L) and (Scott x PD2L) for oat yield by using RAPD markers. Hittalmani *et al.*, (2002) [12] the DNA from F_1 and BC_1F_1 individual was extracted to diagnose for the parents of resistance gene.

Conclusion

Abiotic stress are the major factors that highly affect the production of rice in Asia so there is dire need to develop the variety resistant to those environment. The present study conclude that the genotype NDR 9830102 tolerant to drought and moderatly tolerant to submergence stress condition while Swarna sub 1 and Swarna are high yielding genotype but both were showing susceptibility in drought stress condition. Genotype Swarna sub 1, Swarna and NDR 102 showed genetic diversity among each other on 15% SDS-PAGE gel. Thus by breeding programme crosses have been made between Swarna sub1 x NDR 9830102 and their generated F_1were validated by RAPD primer OPA 11. In future backcross breeding programme will be used to develop a variety resistant to both drought and submergence.

Acknowledgement
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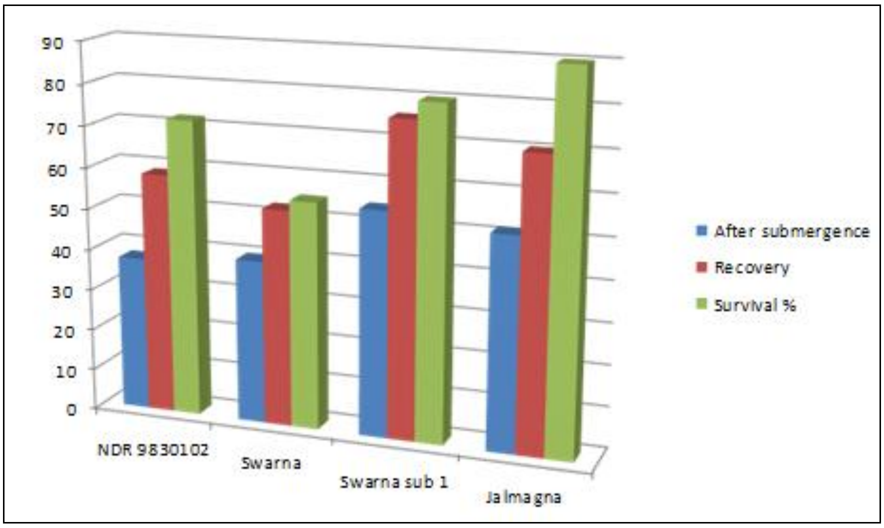
Table 1: Plant height, elongation of shoot and survival percentage due to 14 days of submergence

Genotype	B.S.	Just after de submergence			Shoot elongation			Survival%		
		Control set	Submergence set	Mean	Control set	Submergence set	Mean	Control set Angular Transformed	Submergence set Angular Transformed	Mean
NDR 9830102	81.40	91.77	98.50	95.14	9.81	17.10	13.46	100	70	85.00
Swarna	63.13	67.90	74.23	71.07	4.77	11.10	7.94	100	45	72.50
Swarna sub 1	59.14	63.67	65.20	64.44	4.53	6.06	5.30	100	90	95.00
Jalmagna	97.91	103.52	116.42	109.97	5.61	18.51	12.06	100	90	95.00
Mean		81.72	88.59	85.15	6.18	13.19	9.69	100	73.75	86.88
CD at 5%	7.31	S=7.96, V=5.62, SxV= NS			S=0.87, V=0.61, SxV= 1.23			S=3.87, V=2.74, SxV= 5.48		

Table 2: Effect of submergence on total soluble sugar (mg/ml) of rice genotype

Genotype	Just after de submergence			At recovery		
	Control set	Submergence set	Mean	Control set	Submergence set	Mean
NDR 9830102	76.80	37.50	57.15	88.80	58.50	73.65
Swarna	82.47	39.70	61.09	92.52	52.50	72.51
Swarna sub 1	87.00	54.50	70.75	95.58	75.80	85.69
Jalmagna	76.30	51.50	63.90	86.40	70.25	78.33
Mean	77.65	42.82	60.24	88.08	60.23	74.16
CD at 5%	S=3.64, V=5.75, SxV= 8.14			S=4.51, V=7.13, SxV=10.08		



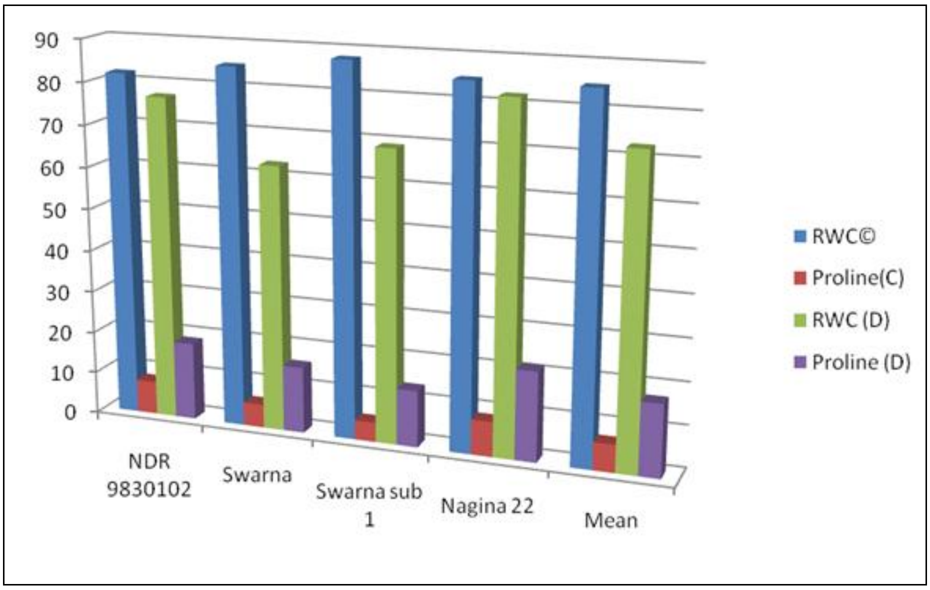
Effect of total solouble sugar on survival percentage.

Fig 1: Genotypes NDR 9830102, Swarna sub 1, Swarna and Jalmagna (Control)

Fig 2: Genotypes Swarna, NDR 9830102, Swarna sub 1 and Jalmagna (After submergence)

Table 3: Effect of drought on the physiological characterstics of rice genotype

Genotype	Plant height (cm)		Tiller/Plant		Number of grains per panicle		Spikelet fertility		Grain Yield	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
NDR 9830102	114.3	103.4	19	17	115.7	95.7	82.3	78.2	20.8	18.5
Swarna	93	81.8	19.3	16	185.6	149.3	85.4	77.4	25.7	22.8
Swarna sub1	90.4	80.6	20.5	16.3	197.0	158.7	83.9	76.0	27.7	23
Nagina 22	109.8	98.6	10.0	8.9	127.3	108.3	86.8	84.8	19.2	17.8
Mean	101.9	91.10	17.2	14.55	156.40	128	84.6	79.1	23.35	20.53
CD at 5%	V=9.29, T=6.56, VxT=13.13		V=1.02, T=0.72, VxT=1.44		V=10.28, T=7.27, VxT=14.54		V=7.80, T=5.52, VxT=11.04		V=1.84, T=1.30, VxT=2.60	



Effect of drought on RWC and Proline content of rice genotypes.

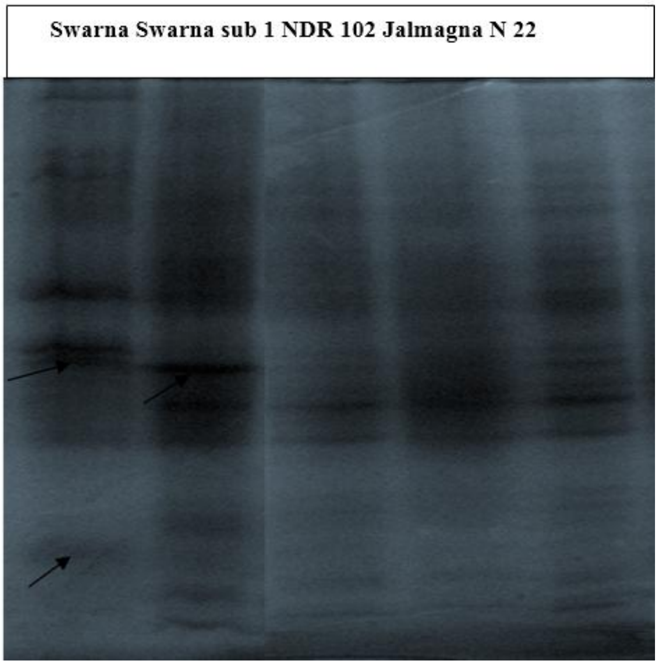


Fig 3: Protein profile of rice genotypes. Lane 1 Swarna. Lanes 2 Swarna sub 1, 3 NDR 102, 4 Jalmagna and 5 Nagina 22, respectively, represent protein samples extracted from leaves of plants. Equal amount of protein (70 lg) were loaded in each lane.

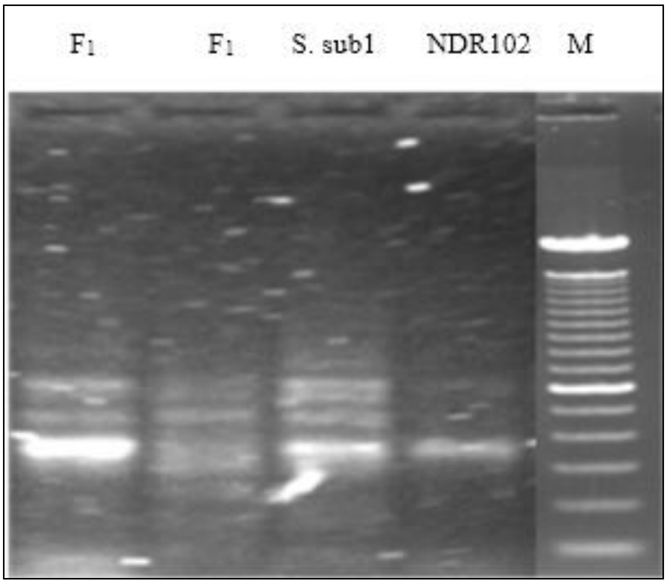


Fig 4: Amplification of genomic DNA of parent (NDR 102, Swarna sub 1) and their F_1s with primer OPA 11. Gel was electrophorsed on 1.5% agrose gel and stained with Et-Br. Marker (100 bp)

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