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Regeneration of rice via somatic embryogenesis and variant analysis using RAPD

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ABSTRACT

RAPD analysis was performed among three rice somaclonal families known to vary for specific characters. Polymorphisms were found among the somaclonal families, All somaclonal families differed significantly from the parental material, indicating that genomic alterations occurred in all families regardless of phenotype. The rice varieties BPT, JJL and Vijaya masuri, among Vijaya masuri showed indirect somatic embryogenesis (calli formation), with 2, 4-D. Good healthy calli was formed at the concentration of 1mg/l, 1.25mg/l, 1.5mg/l and 2mg/l of 2, 4-D. The embyogenic calli so obtained was subcultured into regeneration media containing different concentrations of cytokinins like BAP and Kinetin for getting multiple shoots.

KEYWORDS

RAPD;
Oryzae sativa;
BPT;
JJL;
Vijaya masuri.

INTRODUCTION

Agriculture is the main occupation in India and it is a backbone of our country. But due to variations in monsoon and various stresses formers are facing so many problems which results in decrease of per capita food production with increasing human population and pollution. *O.sativa* L., which constitutes an important part of the diet of more than half of the world's population. As indicated by many workers[15,17,4,12]. The conventional breeding methods are the most widely used for crop improvement. In contrast, the genetic variability and population genetic structure of natural populations of wild rice are less well known[15,4,1,9,6] But in certain situations, these methods have to be supplemented with plant tissue culture techniques either to increase their efficiency or to be able to achieve the objective which is not possible through the conventional methods. The RAPD technique has several advantages over isozyme and other DNA marker methodologies, such as speed, low cost, and the use of small amounts of plant material[10,11]. In recent years, RAPD analysis has become a popular method for estimating genetic diversity and relatedness in plant populations, cultivars and germplasm accessions[11,18,12,16,6]. With this background regeneration of rice via direct and indirect somatic embryogenesis have been choosen for the present study in order to know genetic variation among different species of particular organism and somatic embryo production for genetic manipulation and crop improvement. The

present work is an attempt to raise rice cultivars with different growth regulators via Somatic embryogenesis.

MATEREIALS AND METHODS

Plant material

Rice (*Oryza sativa* L. subtype indica) seeds, varieties including JJL, BPT5204, Vijaya masuri, was obtained from Mahyco seeds company, Karnataka, INDIA were used for micropropagation and variant analysis. The rice seeds were first germinated on Murashige and Skoog (MS 1962) supplemented with 3% sucrose and 1% agar as supporting medium (sigma India). Culture condition was 25±2^{0}C air temperature and 16 hr. Photo period of light intensity 3000 to 5000 lux provided with cool florescent tube lights.

After culturing for 15 days the cotyledons were excised and sub-cultured on MS medium supplemented with different growth hormones namely BAP, KN, BAP+NAA, KN+NAA, BAP+IAA, KN+IAA at varying concentration. The Cotyledons were also inoculated into different types of media namely MS(Full strength), N6 Media, MS(Half strength)+ tryptophan, MS(Half strength) + yeast extract, all of these were modified with different concentration of 2,4-D. The bottles were kept in dark for 30 days for callus formation and then kept in light for 2 weeks. The growth hormones which gave maximum regeneration with somatic embryos formation was selected and again subcultured in MS media containing different concentration of BAP, KN, IAA, NAA.

Establishment of seed culture

Healthy and normal looking paddy seeds were selected and dehusked. Seeds were disinfected with 2% savlon along with 2 to 4 drops of tween 20 by vigorous shaking for 10 to 15 minutes. Washed with running water.All the cultures were incubated at temperature of 25°C and 50-65% relative humidity. The cultures were provided with 16 hours Photoperiod receiving a light intensity of 3000-5000 lux provided with 100 florescent lights from a distance of 30-35cms.

Subculturing conditions

Selective sub-culturing of white, compact embryogenic tissues led to optimized cultures with potential for long-term culture. These embryogenic cultures maintained on MS medium with kinetin, BAP, NAA+BAP, NAA+KN, IAA+KN, IAA+BAP. On other media (media with 0.2mg/1 2,4-D) the frequency of somatic embryogenesis was maintained. Green plantlets regenerated from these cultures grew normally up to maturity. After 4 weeks of incubation healthy rooted plantlets were obtained.

RAPD

Genomic DNA was extracted using a modification of the protocol of Doyle and Doyle[7]. During the DNA fingerprinting through RAPD marker of 3 rice varieties (BPT5204, JJL, VM) we have used the following OPAB primers.

Primer 5'-3'	Sequence
OPAB 05	CCCGAAGCGA
OPAB 09	GGCGACTAC
OPAB 17	TCGCATCCAG

RESULTS AND DISCUSSIONS

1. Effect of N6 media and 2, 4-D on callus induction in rice (Var : BPT5204, JJL, VM)

After inoculating the seeds into the culture bottles containing N6 media with 2,4-D they were incubated in dark for fifteen days for callus induction. At the end of this period, most of the seeds showed the formation of callus. Next they were incubated in light for six to seven days, by this time most of them showed the development of callus they were further sub-cultured.

Observation: After 15 days of incubation the following conclusions were seen.

TABLE 1

Sl.	Conc.	BPT 5204		JJL		VM	
no.	Of 2,4D	Calli	Rate	Calli	Rate	Calli	Rate
1	0.5	Formed	+	Formed	+	Formed	+ +
2	1	Formed	+	Formed	+	Formed	+
3	1.25	Formed	+	Formed	+	Formed	+
4	1.5	Formed	+	-	-	Formed	+ +
5	2	Formed	+	Formed	+	Formed	+

BPT5204: It showed poor development of callus at the concentrations; **JJL:** It showed poor development of callus at the concentrations; **Vijaya masuri:** Concentrations like 0.5 mg/l and 1.5mg/l showed callus these were further subcultured; **Result:** Vijaya masuri showed better response when compared to the other

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two varieties.

2. Effect of tryptophan in MS media (half strength) and 2, 4-D on callus induction in rice(Var: BPT5204, JJL, VM)

After inoculating the seeds into the culture bottles containing MS-2S media with tryptophan and 2,4-D they were incubated in dark for fifteen days for callus induction. At the end of this period, most of the seeds showed the formation of callus. Next they were incubated in light for six to seven days; by this time most of them showed the development of callus they were further sub-cultured.

Observation: After 15 days of incubation the following conclusions were seen.

TABLE 2

Sl. no.	Conc. Of 2,4D	BPT 5204 Calli	BPT 5204 Rate	JJL Calli	JJL Rate	VM Calli	VM Rate
1	0.5	Formed	+	Formed	+	Formed	+ +
2	1	Formed	+	Formed	++	Formed	++
3	1.25	Formed	+	Formed	++	Formed	++
4	1.5	Formed	++	Formed	+	Formed	+ +
5	2	Formed	+	Formed	+	Formed	+++

BPT5204: showed normal development of callus. At the concentration 1.5mg/l better conclusions were seen; **JJL:** concentrations like lmg/l and 1.25mg/l showed callus these were further subcultured; **Vijaya masuri:** Concentrations like 2 mg/l 2,4-D showed callus these were further subcultured; **Result:** Vijaya masuri showed better response at the concentrations of 2mg/l when compared to other varieties.

3. Effect 0f yeast in MS media (half strength) and 2, 4-D on callus induction in rice (Var: BPT5204, JJL, VM)

After inoculating the seeds into the culture bottles containing MS-2S media with 2,4D they were incubated in dark for fifteen days for callus induction. At the end of this period, most of the seeds showed the formation of callus. Next they were incubated in light for six to seven days; by this time most of them showed the development of callus they were further sub-cultured.

Observation: After 15 days of incubation the following conclusions were seen.

BPT5204: At the concentration like 1.5mg/l 2, 4 - D showed callus these were further sub cultured; **JJL:** It showed poor callus formation at all the concentrations;

TABLE 3

Sl. no.	Conc. Of 2,4D	BPT 5204 Calli	BPT 5204 Rate	JJL Calli	JJL Rate	VM Calli	VM Rate
1	0.5	Formed	+	Formed	+	Formed	+
2	1	Formed	+	Formed	+	Formed	++
3	1.25	Formed	+	Formed	+	Formed	++
4	1.5	Formed	++	Formed	+	Formed	+ +
5	2	-	-	Formed	+	Formed	++

Vijaya masuri: Concentrations like l mg/l, 1.25mg/l, 1.5mg/l and 2 mg/l 2,4 D showed callus these were further subcultured; **Result:** Vijaya masuri showed better response when compared to the other varieties.

4. Effect of ms-2s media and 2,4d on callus induction in rice (Var: BPT5204, JJL, VM)

After inoculating the seeds into the culture bottles containing MS-2S media with 2,4D they were incubated in dark for fifteen days for callus induction. At the end of this period, most of the seeds showed the formation of callus. Next they were incubated in light for six to seven days, by this time most of them showed the development of callus they were further sub-cultured.

Observation: After 15 days of incubation the following conclusions were seen.

TABLE 4

Sl. no.	Conc. Of 2,4D	BPT 5204 Calli	BPT 5204 Rate	JJL Calli	JJL Rate	VM Calli	VM Rate
1	0.5	Formed	+++	Formed	+++	Formed	+ +
2	1	Formed	+++	Formed	+++	Formed	+++
3	1.25	Formed	+++	Formed	++	Formed	+++
4	1.5	Formed	+++	Formed	+++	Formed	+++
5	2	Formed	++	Formed	++	Formed	+++

BPT5204: Some concentrations like 0.5mg/l, 1mg/l, 1.25mg/l, 1.5mg/l 2,4 D showed callus these were further subcultured; **JJL:** Concentrations like 0.5mg/l, l mg/l and 1.5mg/l 2,4 D showed callus these were further subcultured; **Vijaya masuri:** Concentrations like lmg\l, 1.25mg/l, 1.5mg/l and 2 mg\l 2,4 D showed callus; these were further subcultured; **Result:** All the varieties of rice showed better response at the concentrations l mg/1 and 1.5 mg/1 of 2,4 D.

5. Effect of BAP on cotyledons culture of Oryza sativa (Var: JJL, BPT5204, Vijaya masuri) MS-2S full strength media

After inoculating the seeds into the culture bottles containing MS-2S, they were incubated in dark for five days to germinate. By the end of this period, most of

the seeds had germinated. Next they were incubated in light for six to seven days, by this time both shoot and root was seen. The seedlings were then taken out in aseptic condition and the cotyledons were cut. These cotyledons were inoculated into media containing different concentrations of BAP.

Observation: After 15 days of incubation the following conclusions were seen

TABLE 5

Sl. no	Cone of BAP	BPT 5204		JJL		VM	
		Observation	Rate	Observation	rate	Observation	rate
1	0.5	Multiple shoots	+ +	Multiple shoots	+ +	Multiple shoots	+ +
2	1.0	Single shoot	+ +	Multipleshoots	+ + +	Single shoot	+
3	1.25	Multiple shoots	+ + +	Multiple shoots	+ + +		
4	1.5	Multiple shoots	+ + +	Single shoot	+ + +	Multiple shoots	+
5	2.0	Multiple shoots	+ + +	Multiple shoots	+ +	Multiple shoots	+ + +

BPT5204: Some concentrations like 1.25mg/l, 1.5 mg/l and 2.0mg/l BAP showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **JJL:** Some concentrations like l.0mgM and 1.25mg/l BAP showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Vijaya masuri:** Some concentrations like 2.0 mg/l BAP showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Result:** The effect of BAP was seen much better in BPT 5204 than other varities.

6. Effect of kinetin on cotyledons culture of *Oryza sativa* (Var: JJL, BPT5204,Vijaya masuri) MS-2S full strength media

After inoculating the seeds into the culture bottles containing MS-2S, they were incubated in dark for five days to germinate. At the end of this period, most of the seeds had germinated. Next they were incubated in light for six to seven days, by this time both shoot and root was seen. The seedlings were then taken out in aseptic condition and the cotyledons were cut. These cotyledons were inoculated in to media containing different concentration of Kinetin.

Observation : After 15 days of incubation the following response were seen.

TABLE 6

Sl. no	Conc. of Kinetin	BPT 5204		JJL		VM	
		Observation	rate	Observation	rate	Observation	rate
1	0.1			Single shoot	+ + +		
2	0.2	Single shoot	+ + +	Single shoot	+ +	Multiple shoots	+ + +
3	0.3	Single shoot	+ + +	Multiple shoots	+ + +	Single shoot	+
4	0.4	Multiple shoots	+ +	Single shoot	+ +	Single shoot	+ + +
5	0.5	Single shoot	+ + +	Single shoot	+ + +	Single shoot	+ + +

BPT5204: Some concentrations like 0.4mg/l Kinetin showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **JJL:** Some concentrations like 0.3mg/l Kinetin showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Vijaya masuri:** Some concentrations like 0.2 mg/l Kinetin showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Result:** The effect of Kinetin was seen much better in JJL and VM.

7. Effect of IAA and Kinetin on cotyledons culture of Oryza sativa (Var: JJL, BPT5204, Vijaya masuri) MS-2S full strength media

After inoculating the seeds into the culture bottles containing MS-2S, they were incubated in dark for five days to germinate. At the end of this period, most of the seeds had germinated. Next they were incubated in light for six to seven days, by this time both shoot and root was seen. The seedlings were then taken out in aseptic condition and the cotyledons were cut. These cotyledons were inoculated in to media containing different concentrations of IAA and Kinetin.

Observation: After 15 days of incubation the following conclusions were seen

TABLE 7

Sl. no.	Conc. of IAA and Kinetin	BPT 5204		JJL		VM	
		Observation	rate	Observation	rate	Observation	rate
1	0.01+5	Multiple shoots	+	Single shoot	+ +	Single shoot	+ +
2	0.01+1	Single shoot	+ + +	Multiple shoots	+ +	-	
3	0.05+1	-		Multiple shoots	+ +	Single shoot	+ +
4	0.5+1	Multiple shoots	+ +	Single shoot	+ +	Single shoot	+ + +
5	0.5+2	Multiple shoots	+	Multiple shoots	+ +	Multiple shoots	+

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BPT5204: Some concentrations like 0.5+lmg/l IAA & Kinetin showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **JJL:** Some concentrations like 0.01+lmg/l ,0.05+lmg/l and 0.5+2 mg/l IAA & Kinetin showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Vijaya masuri:** Some concentrations like 0.5+2mg/l IAA & Kinetin showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Result:** The effect of IAA and Kinetin was seen much better in BPT and JJL.

8. Effect of IAA and BAP on cotyledons culture of *Oryza sativa* (Var: JJL, BPT5204,Vijaya masuri) MS-2S full strength media

After inoculating the seeds into the culture bottles containing MS-2S, they were incubated in dark for five days to germinate. At the end of this period, most of the seeds had germinated. Next they were incubated in light for six to seven days, by this time both shoot and root was seen. The seedlings were then taken out in aseptic condition and the cotyledons were cut. These cotyledons were inoculated in to media containing different concentrations of IAA and BAP.

Observation: After 15 days of incubation the following conclusions were seen.

TABLE 8

Sl. no	Conc. of IAA and BAP	BPT 5204		JJL		VM	
		Observation	rate	Observation	Rate	Observation	rate
1	0.5+2	Multiple shoots	+	Multiple shoots	+ + +	Multiple shoots	+ +
2	0.1+2	Multiple shoots	+ + +	Multiple shoots	+ + +	Single shoot	+
3	0.1+2	Single shoot	+	Multiple shoots	+ +	Single shoot	+ +
4	0.01+1	Multiple shoots	+ +	Single shoot	+	Multiple shoots	+ +
5	0.05+1	Multiple shoots	+ ++	Multiple shoots	+ +	Multiple shoots	+

BPT5204: Some concentrations like 0.1+2mg/l, 0.05+1mg\l showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **JJL:** Some concentrations like 0.5+2mg/l, 0.1+2mg/l IAA & BAP showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Vijaya masuri:** Some concentrations like 0.5+2mg/l and 0.01 + l mg/l IAA & BAP showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Result:** The effect of BAP and IAA was seen much better in BPT 5204 and JJL.

9. Effect of BAP and Kinetin on cotyledons culture of *Oryza sativa* (Var: JJL, BPT5204,Vijaya masuri) MS-2S full strength media.

After inoculating the seeds into the culture bottles containing MS-2S, they were incubated in dark for five days to germinate. At the end of this period, most of the seeds had germinated. Next they were incubated in light for six to seven days, by this time both shoot and root was seen. The seedlings were then taken out in aseptic condition and the cotyledons were cut. These cotyledons were inoculated in to media containing different concentrations of BAP and Kinetin.

Observation : After 15 days of incubation the following conclusions were seen

TABLE 9

Sl. no.	Conc. of BAP and Kinetin	BPT 5204		JJL		VM	
		Observation	rate	Observation	rate	Observation	rate
1	0.01+0.5	Multiple shoots	+	Single shoot	+ + +	Single shoot	+ +
2	0.01 + 1	Multiple shoots	+ + +	Multiple shoots	+ +	Multiple shoots	+ +
3	0.05+1	--	--	Single shoot	+	Multiple shoots	+ +
4	0.5+1	Single shoot	+ + +	Single shoot	+++	Multiple shoots	+ +
5	0.5+2	Single shoot	+ +	Single shoot	+ + +	Single shoot	+

BPT5204: Some concentrations like 0.01+lmg/l BAP & Kinetin showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **JJL:** Some concentrations like 0.01+1mg/l BAP & Kinetin showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Vijaya masuri:** Some concentrations like 0.01+lmg/l, 0.05+lmg/l and 0.5+1mg/l BAP & Kinetin showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Result:** The effect of BAP and Kinetin was seen much better in VM and BPT 5204.

10. Effect of NAA and BAP on cotyledons culture of *Oryza sativa* using MS-2S full strength media. (Var: JJL, BPT5204, Vijaya masuri)

After inoculating the seeds into the culture bottles containing MS-2S, they were incubated in dark for five days to germinate. At the end of this period, most of the seeds had germinated. Next they were incubated in light for six to seven days, by this time both shoot and root was seen. The seedlings were then taken out in aseptic condition and the cotyledons were cut. These cotyledons were inoculated in to media containing different concentrations of NAA and BAP.

Observation: After 15 days of incubation the following response were seen.

TABLE 10

Sl. no.	Conc. of NAA and BAP	BPT 5204		JJL		VM	
		Observation	rate	Observation	rate	Observation	rate
1	0.5+1	Single shoot	+ +	Multiple shoots	+ +	-	-
2	0.1 + 1	Single shoot	+ +	Multiple shoots	+ +	-	-
3	0.1+2	Multiple shoots	+ +	Multiple shoots	+ +	-	-
4	0.01+1	Multiple shoots	+ +	Single shoot	+ +	Multiple shoots	+ + +
3	0.05+1	-	-	Single shoot	+	Multiple shoots	+ +

BPT 5204: Some concentrations like 0.1+2mg/l and 0.01 + 1 mg/l NAA & BAP showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **JJL:** Some concentrations like 0.5+lmg/l, 0.1+lmg/l and 0. l+2mg/l NAA & BAP showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Vijaya masuri:** Some concentrations like 0.01+lmg/l and 0.05mg/l NAA & BAP showed direct embryogenesis through formation of multiple shoots. These shoots were further subcultured into auxin containing media for rooting; **Result:** The effect of NAA and BAP was seen much better in three varieties also.

OPAB-05

Observation : All the three varieties are having a single prominent band which are monomorphic. So, the varieties cannot be differentiated.

Result: OPAB-05 cannot be used as a differential marker to distinguish the three varieties.

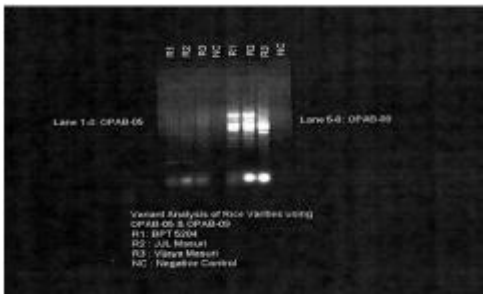

Figure: 10.1

OPAB-09

Observation: R1 variety is having 10 bands, R2 variety is also having 10 bands and R3 variety is having 5 bands. In which 3 monomorphic bands are present in all the 3 varieties. Apart from these 2 similar bands are present from top in the varieties R1 and R2 which can be differentiated from R3.

Result : OPAB-09 primer can be used as a differential marker to distinguish R1 and R2 from R3 variety.

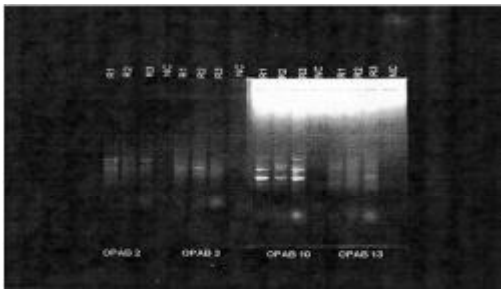

Figure: 10.2

OPAB 02

Observation : R1 variety is having 6 bands, R2 variety is having 7 bands and R3 is also having 7 bands. In which 2 monomorphic bands are present in Rl, R2 and R3. Apart from these 2 bands 2 different bands are present from the top in R3 variety.

Result : OPAB 02 primer can be used as a differential marker to distinguish R1 and R2 from R3 variety.

OPAB 03:

Observation : R 1 variety is having 3 bands, R 2 variety is having 5 bands and R 3 variety is having 3 bands. In which 1 monomorphic band is present in R l, R 2 and R 3. Apart from these 1 prominent is present in R 2 variety which is absent in other 2 varieties.

Result: OPAB 03 primer can be used as a differential

marker to distinguish R1 and R3 from R2 variety.

OPAB 10

Observation: R1 variety is having 9 bands, R2 variety is having 8 bands and R3 variety is having 9 bands. In which 2 monomorphic bands are present in R1, R2 and R3. Apart from these 2 prominent is present in R1 and R3 varieties which is absent in R 3 variety.

Result: OPAB 10 primer can be used as a differential marker to distinguish R2 variety from R1 and R3 varieties.

OPAB 13

Observation: R1 variety is having 8 bands, R2 variety is having bands but which are not clear and R3 variety is having 6 bands. Among R1 and R3, 2 monomorphic bands are present. Apart from these 1 prominent is present in R3 which can be distinguished from R 1.

Result: OPAB 13 primer cannot be used as a differential marker to distinguish the 3 varieties. But R3 variety can be distinguished from R1.

CONCLUSION

From the above results we can conclude that *Vijaya masuri* has responded well in all the media when compared to BPT 5204 and JJL. MS-2S media with 2,4D showed better response when compared to other media. Micro-propagation is a technique where a callus mass has been initiated from a single explant taken from any living part of a donor plant and within a very short time and space, a large number of plantlets can be produced from such callus tissue. Further it is possible to make large number callus pieces from the original stock culture through sub-culturing. The establishment of micro-propagation for rapid propagation, the use of shoot tip culture to produce nuclear stock free from parasites especially viruses and genetic manipulation have contributed to the acceptance of plant tissue culture as valuable tool for plant improvement. Large number of plant species can be propagated all the year round i.e., the plant breeder is no longer restricted by season in the production of large numbers of plants. The conventional breeding methods are the most widely used for crop improvement. But in certain situations, these methods have to be supplemented with plant tissue culture techniques either to increase their efficiency or to be able to achieve the objective which is not possible through the conventional methods.

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