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COMPARATIVE STUDIES OF *In vitro* AND *In vivo* RAISED SEEDLINGS OF *Oryza sativa* L.

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ABSTRACT

Seeds of three Indian rice (*Oryza sativa* L.) varieties Sita, Rupali and Swarna Masuri were evaluated for in *vitro* (using MS media supplemented with 0.5mg/l IAA) and in *vivo* (using water soaked cotton) germination. Comparison of those germinated seedlings on the basis of in *vivo* and in *vitro* condition by using morphological, biochemical and analytical parameter were done. Seeds were used as explant source on both the culture condition. In morphological study, analysing growth rate i.e the length of geminating seedlings, best response were observed for in *vitro* ones. However, both the culture conditions were not found to be differentiate in the viability frequency of varities and they resulted more or less equal. In contrast to biochemical study, the in *vitro* varieties were seen to be rich in chlorophyll and starch content whereas the in *vivo* were higher in total phenolic content and more potential in catalase and peroxidase enzyme activity. The ability of accumulation of protein was imprecisely same for all three varities that grown up on different culture condition. Spectrophotometric estimation of starch and total phenolic were became more clarified when they showed same supportive result in analytical study of thin layer chromatography. Native gel electrophoresis was performed for esterase isozyme by result which only differ in their concentration but not in any bands.

KEYWORDS : *Oryza sativa*, In *vitro*, Biochemical Comparison, TLC, Native-PAGE, Tissue Culture

Rice is an important cereal crops and it provides as much as 80% of the required calories for the people in West Bengal. In much of Asia, rice is so central to the culture that the world is almost synonymous with food (Alam et al, 2012). A considerable improvement has been done through traditional rice breeding. Rice breeding has made significant progress towards higher yield, improved quality, greater disease resistance and other important characters of agricultural importance in the past and it will still play an important role (Sun et al., 1990). International Rice Research Institute (IRRI) is employing several tissue culture techniques to develop rice varieties (Bajaj et al, 1996). Various tissue culture techniques are being applied for varietal development of cereal crops including rice in different countries (Dorosieve et al, 1996). Among which dehusked seed culture is a valuable and non-conventional method to exploit somaclonal variation, disease resistant, increasing its nutritional quality for creation of a novel rice varieties within a very short period (Alam et al, 2012). In *vitro* culture of plant cells and tissue has attracted considerable interest over recent years because it provides the mean to study plant physiological, biochemical and genetic processes in addition to offering the potential to assist in the breeding of improved cultivars by increasing genetic variability (Karp et al, 1987). Sita, Rupali and Swarna Masuri are the most important rice varieties of Asansol, Dist. Burdwan, West Bengal for their fine grain, good test and reasonably priced.

Therefore, this present study is aimed to compare the three commercial rice (*Oryza sativa* L.) cultivars (Sita, Rupali, Swarna Masuri) on the basis of in *vivo* and in *vitro* culture condition using mature seeds as a source of explant. The system consist of two stages, germination and their comparative study through morphological, biochemical and analytical parameter. Today's research is mainly focused on how to increase the nutritional food value of any cereal crops. In this study as the seeds are grown up in different culture condition (in *vivo* and in *vitro*) thus there should be a genetical, morphological or biochemical changes will occur. My objective is to determine those changes at seedling stage and tabulate them as a comparative form.

MATERIALS AND METHODS

Healthy seeds of *Oryza sativa* L.(family Poaceae) of three varieties viz. Sita, Rupali and Swarna Masuri MTU-7029 also known as Masuri were collected from Agricultural Blok Office, Jamuria-II, Burdwan (West Bengal), collected seeds were authenticated from Assistant Director of Agriculture.

In *vitro* germination

Manually dehusked seeds were treated with 2% extran for 10 minutes and washed thrice in sterile distilled

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water. Seeds were surface sterilized in 0.1% mercuric chloride ($HgCl_2$) for 5-7 minutes followed by three rinses in sterile distilled water in a laminar flow cabinet. Sterilized seeds were cultured in bottles containing semisolid MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/l IAA and allowed to grow for 10 days inside the culture room which is maintained at ±27°C, 70-75% humidity and 12 hours light and 12 hours of dark cycle.

In *vivo* germination

Water soaked(for 48 hours) seeds were spread over the Petri plates beds (prepared using blotting paper followed by absorbent cotton) and allowed to germinate under appropriate sunlight, temperature and humidity for 10 days.

Estimation of chlorophyll content

The chlorophyll content of the seedlings were estimated according to the method of Arnon, 1949 and the amount of total chlorophyll was calculated using formula given below:

$$\text{Total chlorophyll} = \frac{[20.2\ (D645) + 8.02(D663)]V}{1000\ (W)}$$

[D = Optical density value, V = Final volume of 80% alkaline acetone, W = Fresh weight in g of tissue extract]. The total chlorophyll content was estimated in the term of mg of chlorophyll/g of fresh tissue.

Estimation of starch content

The starch content of the seedlings were done according to Lohar et al, 2010 [by the spectrophotometric method of Mont Gomery, 1957].

TLC for starch

TLC for separation of starch were done according to Abere et al, 2005 with some modification. Seedlings were extracted with 80% ethanol. Whatman chromatographic paper (that activated at temperature of 110°C for 1 hour) used as a stationary phase and Butanol : Glacial acetic acid : water :: 5 : 2 : 3 was as mobile phase. Evaporated supernatants were used as a sample. Earlier saturated iodine tank was used as a spot locating agent.

Estimation of total phenolic content

Total phenolic content of germinated seedlings were determined according to Nataraj et al, 2009 and Mohammed et al, 2013. Reaction mixture was prepared by 0.25ml of each extract (100mg/ml), 0.5ml Folin- Ciocalteau and 2.25 ml distilled water. After 3 minutes, 2ml of 10% sodium carbonate (Na_2CO_3) was added and the mixture was incubated in boiling water for 30 seconds and allowed to stand for 30 mins at 40°C for colour development. Absorbance was then measured at 650nm wavelength on a spectrophotometer. TPC of different extracts were measured from the standard curve of stock solution of catechol (100µg/ml).

TLC for phenolic compound

TLC for phenolic compound of germinated seedlings were done according to Prabhu et al, 2011 with some modification. Seedlings were extracted with 80% ethanol. Aliquot of supernatant were dried properly in a hot water bath until it evaporates to dryness. Butanol : Glacial acetic acid : water :: 4 : 1 : 5 was used as mobile solution and Whatman chromatographic paper as stationary phase. Folin-Ciocalteau reagent followed by 20% Na_2CO_3 were used as visualizing agents.

Estimation of protein content

Estimation of protein content of in *vivo* and in *vitro* seedlings were done according to Lohar et al, 2010 [by Lowry method (1951)].

Determination of specific activity of catalase

The activity of enzyme catalase assayed by the method of (Casper and Laccope, 1968) with some modification. Seedlings were extracted (100mg/ml) with 0.1M phosphate buffer (pH 7.0). Sets of reaction mixture were prepared at different incubation period by 10ml of 0.1M Phosphate buffer, 1ml of 5% H_2O_2, 5ml of 10% H_2SO_4 and 1ml of enzyme extract. Each set was titrated against N/50 $KMnO_4$ solution, with a faint pink colour (stable for 20 sec.) denoting the end point from which the results were obtained and it was represented in terms of H_2O_2 decomposed by the enzyme ($mg^{-1}\ hr^{-1}$).

Determination of specific activity of peroxidase

The specific activity of enzyme peroxidase of germinated seedlings were determined according to Ling et al, 2013 [by the method of Kokkinakis and Brooks, 1979] with some modification. Seedlings were extracted (100mg/ml) with 0.05M phosphate buffer (pH 6.0)

Reaction mixture were prepared by 5ml of 0.1M Phosphate buffer, 1ml of 3% H2O2, 1ml of 0.5% Catechol and 0.5ml of enzyme extract. For each set absorbance at 420 nm was taken at a time interval of 1 minute and the activity of the enzyme was calculated in respect of change of OD at 420nm/g/min.

Isozyme Electrophoresis

The experiment was done according to Youssef et al, 2011 with some modification. 200 mg fresh seedlings were crushed with 1ml 0.2M Sodium-phosphate buffer (pH 6.0). The supernatants were then separated in 8% native-PAGE at 80 volts for 2 hours. Alpha-napthyl acetate : Acetone : Distilled water : 0.1M Sodium-phosphate buffer pH 6.0 : Fast blue RR salt :: 0.05g : 0.5ml : 0.5ml : 50ml : 0.05g was used as staining solution. Gel was incubated at dark (37°C) for 20-30 minutes. All the events of performing native-PAGE i.e. starting from crushing till staining was done by maintaining the temperature at 4°C.

RESULT AND DISCUSSION

The study of comparison in *vivo* and in *vitro* germinated seedlings begin with the morphological study. The optimum response in case of in *vitro* and in *vivo* germination of seedlings were obtained for all three varieties resulting in the order of highest to lowest according to seed viability is Rupali>Masuri>Sita (Figure 1) and according to the length of germinated seedlings at a interval of day 10 is Masuri>Rupali>Sita (Figure 2). The percentage of seed viability and length of germinated seedlings for both the condition showed same order among the varieties, they only differ in percentage. In *vitro* were showed a higher percentage and frequency than the in *vivo* ones. Its too simple to conclude that in the in *vitro* condition due to its higher availability of nutrition the seeds were accumulate more primary metabolites and storage material due to which viability and growth rate is too high with respect to in *vivo* ones which were grown under natural condition in water soaked cotton by using the grains as their source of nutrition only.

Chlorophyll are the essential components for photosynthesis, and occur in chloroplasts as green pigments in all photosynthetic plant tissue. They are bound loosely to

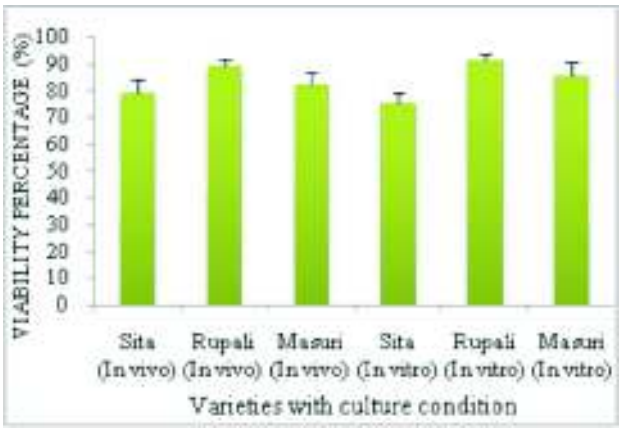


Figure 1 : Effect of in *vivo* and in *vitro* Culture Condition on Seed Viability.

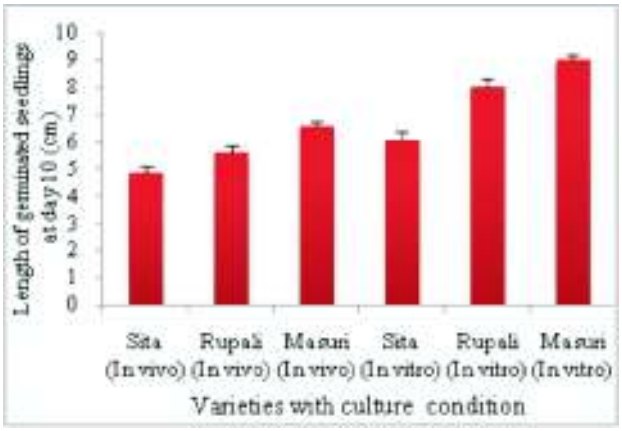


Figure 2 : Effect of in *vivo* and in *vitro* Culture Condition on Length of Germinated Seedlings.

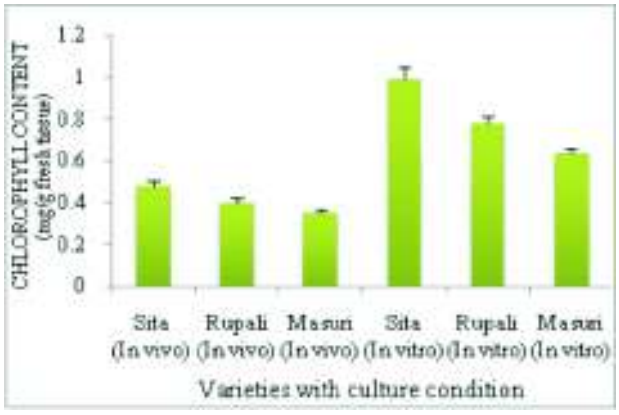


Figure 3 : Effect of in *vivo* and in *vitro* Culture Condition On Total Chlorophyll Content.

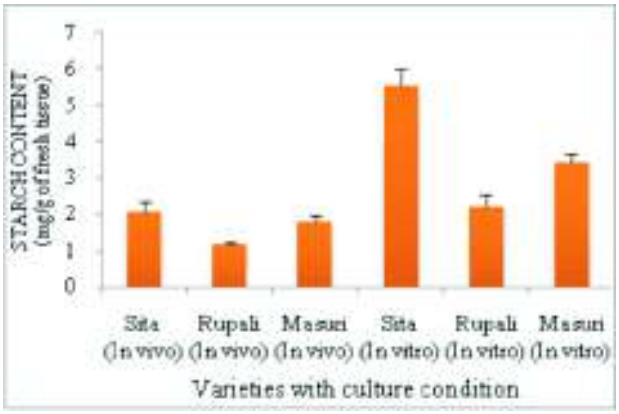


Figure 4 : Effect of in *vivo* and in *vitro* Culture Condition on Starch Content.

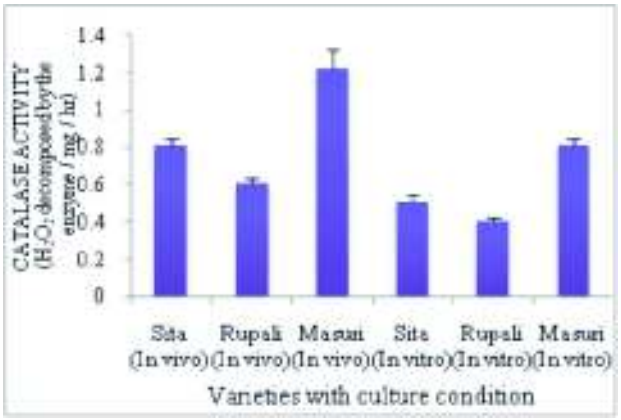


Figure 7 : Effect of in *vivo* and in *vitro* Culture Condition On Catalase Activity

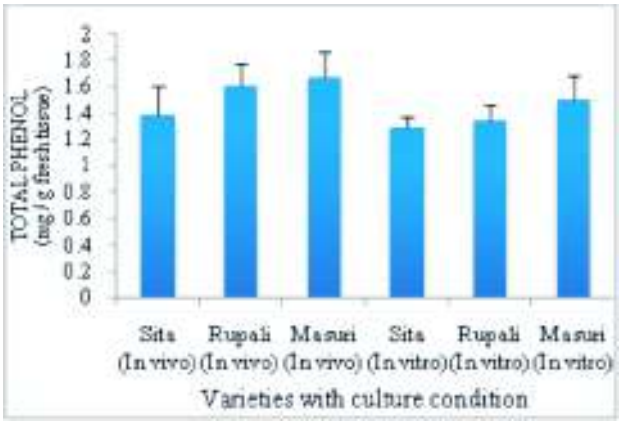


Figure 5 : Effect of in *vivo* and in *vitro* Culture Condition On Total Phenol Content.

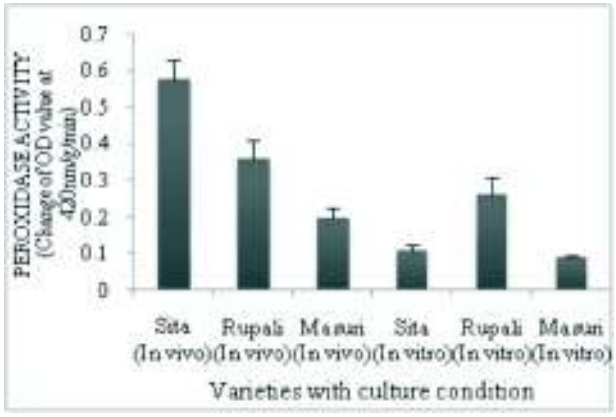


Figure 8 : Effect of in *vivo* and in *vitro* Culture Condition on Peroxidase Activity

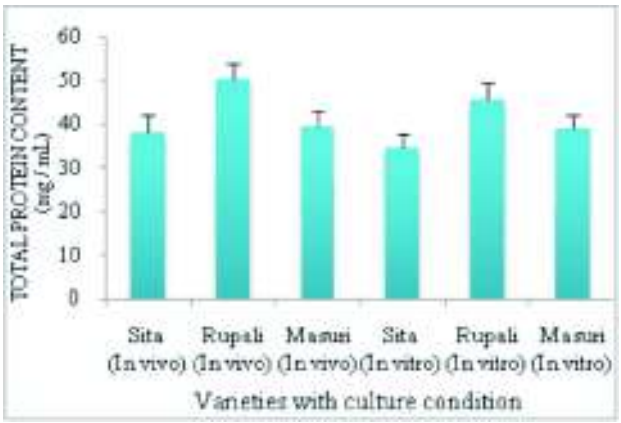


Figure 6 : Effect of in *vivo* and in *vitro* Culture Condition on Total Protein Content

proteins but are readily extracted in organic solvent, among which acetone or ether is the best extraction solvent (Dere et al, 1998). Estimation of chlorophyll and starch content revealed high concentration in case of in *vitro* condition. According to Phonguodume et al., 2012 light plays an important role in the development of plants. They posses maximum growth rate or biomass having larger amount of chlorophyll as well as higher light sources for energy and survival, because chlorophyll content is directly dependent upon the light intensity of its growing environment. Thus, due to controlled light intensity for in *vitro* condition the chlorophyll content is higher compared to in *vivo* (Figure 3). Starch, consists of unbranched molecules of amylose and branched molecules of amylopectin is an important

polysaccharide and is a reservoir of carbohydrate in plants. Starch is solubilised by extracting the plant material in perchloric acid. This solubilised starch when react with hot acidic medium hydrolyses to glucose and dehydrated to hydroxymethyl furfuryl and produce a colour product that was quantified colorimetrically. The in *vitro* culture media is rich of sucrose. As the chlorophyll content is high in in vitro condition the rate of photosynthesis is also more (Figure 4). During photosynthesis, CO_2 and inorganic phosphate are converted into trioseP which accumulate high amylose, resulting more storage of starch (Gibson et al, 2011).

In preliminary screening of total phenolic through quantitative (spectrophotometric estimation) study both the in *vivo* and in vitro were showed more or less same phenolic content but still seeds germinated under in *vivo* condition were a little bit higher than the in vitro ones (Figure 5). Plants are conceived as sources of antioxidants due to presence of polyphenols and flavonoids which possess wide biological properties. Recent studies showed that many flavonoids & related phenols contribute significantly to the total antioxidant and scavenging activity of many plants (Supe et al., 2014). The spectrophotometric reaction for determination of total soluble phenolic content is based on the reduction of phosphomolybdic and phosphotungstic acids from Folin-Ciocalteau reagent by phenolic groups found in the sample.

Protein content of the growing plant mostly depends on the moisture content (Rao et al, 1987). In the present experiment, the estimated protein content was little less in in vitro plants with respect to the in *vivo* plants (Fig. 6). The plants under in *vivo* conditions were continuously supplied with water whereas the in vitro plants are grown in the culture media with varying mineral concentration. This varying mineral concentration and decrease in the moisture content within the media may be the reason for the reduction in the concentration of proteins after certain period. Water deficit in plant tissue may cause reduction in polyribosome levels or lead to increase in the protein breakdown (Sikuku et al, 2010). This implicates that habitat preference which in turn is the key factor that sustains the unique composition of the photochemical.

TLC (Thin Layer Chromatography) is a simple, quick, and inexpensive procedure that gives a quick answer as to how many components and their concentration present in a mixture. Separation of compounds is based on the competition of the solute and the mobile phase for binding places on the stationary phase (Bele et al, 2011). In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried further up the plate than others. In present study, separation of starch and total phenol, done by TLC, showed some distinct bands. Bands were presents more or less in all the varieties at both the culture condition; they only differ at their concentration but not in any number, i.e. supporting the results that obtained through the spectrophotometric estimation.

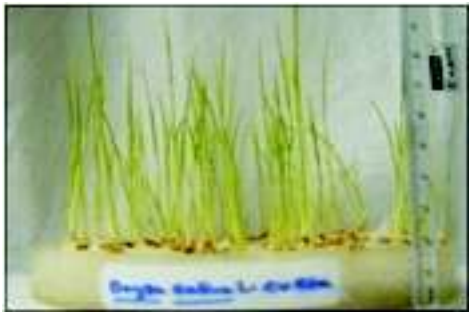
Catalase is an iron containing tetra-pyrolic compound found in nearly all living organism exposed to oxygen which catalyses the decomposition of H_2O_2 to water and oxygen and play a preventional role in damage of any cell or tissue. The other enzyme peroxidase includes in its widest sense a group of specific enzymes such a NAD-Peroxidase, NADP-Peroxidase, fatty acid peroxidase as well as a group of very non-specific enzymes from different sources which catalyzes the dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, hydroquinones etc and seems to have the role in removing potentially harmful oxidising agent. Thus those selected enzymes play an important role in defence responses of plants to biotic or abiotis stresses (Luhova et al, 2003). In the next step of biochemical comparison i.e the assay of specific activity of these two enzymes were done and found to having much more in in *vivo* germinated seedlings. From the obtained result its clarify that both the enzyme present in higher amount in the in *vivo* seedlings. The reason may be due to the environmental condition supplied to the plant material i.e. the in *vivo* seeds are grown by overcoming different environmental factors whereas the in *vitro* were grown under controlled favourable culture condition (Figure 7 and 8).

Enzymes with the same catalytic activity with different molecular forms are known as iso(en)zymes. They are an essential feature of the biochemical organization of

A= Sita (In *vitro*)

B= Rupali (In *vitro*)

C= Masuri (In *vitro*)

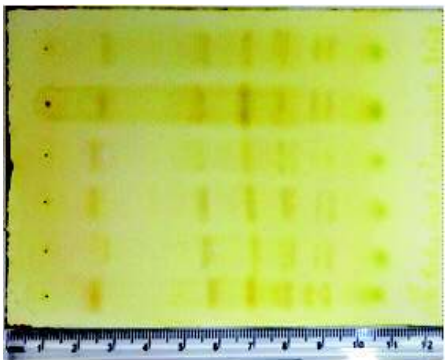


D= Sita (In *vivo*)

E= Rupali (In *vivo*)

F= Masuri (In *vivo*)

Figure 9 : Differences in the Length of Germinated Seedlings on The Basis of Culture Condition at Day 10; A, B, C: Seedlings Grown Under in *vitro* Condition (MS Medium Supplimented With 0.5 Mg/l Iaa); D, E, F: Seedlings Grown Under in *vivo* Condition (water Soaked Cotton).

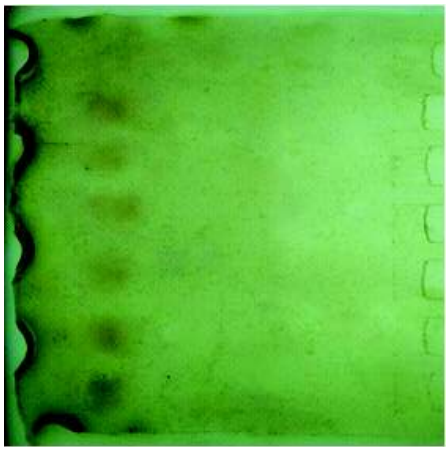


Sita (in *vivo*)
Sita (in *vitro*)

Rupali (in *vivo*)
Rupali (in *vitro*)

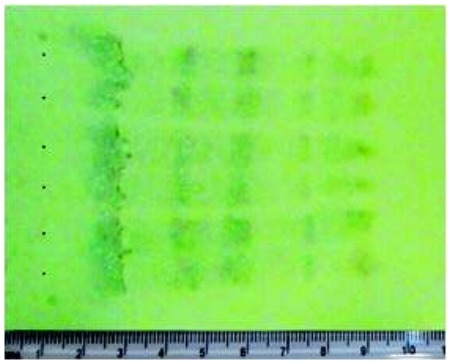
Masuri (in *vivo*)
Masuri (in *vitro*)

A



Dye only
Masuri (in *vivo*)
Masuri (in *vitro*)
Rupali (in *vivo*)
Rupali (in *vitro*)
Sita (in *vivo*)
Sita (in *vitro*)

C



Rupali (in *vivo*)
Rupali (in *vitro*)
Sita (in *vivo*)
Sita (in *vitro*)
Masuri (in *vivo*)
Masuri (in *vitro*)

B

Figure 10. A: TLC For Separation of Starch; B: Separation of Total Phenolics Through TLC; C: Native Page Showing Separation of Esterase Isozyme on The Basis of Charge and Mass Ratio, Observed Under Light Source of Transilluminator.

living things. A number of major biological problems in differentiated tissues are understood in the light of isozyme. They may arise due to micro heterogeneity in proteins, changes in conformation, genetic mechanisms etc. Native-polyacrylamide gel electrophoresis (Native-PAGE) separates proteins primarily by their charge-to-mass ratio and in their native conformations. The isozyme esterase was present more or less within all varieties of *Oryza sativa* (Gaszmann et al, 1987), thus it was alloweded for electrophoretic separation. From the result obtained it was possible to identify them at their native condition which gives distinct bands at a particular molecular weight within the PAGE resulting to differ only in their concentration.

CONCLUSION

Present study reported a successful *sativa* rice high frequency direct germination protocol from mature seed in three diverse and highly productive cultivars Sita, Rupali and Swarna Masuri belonging to Indian subcontinent, which showed yield potential of in *vitro* developed seedlings at par to that of direct seeded seedlings in ambient condition. Although rice is not among the foods with higher concentrations of plant phenolics and protein content and its not taken out for those, still the similarity in concentration of those component on both condition is beneficial to exploit somaclonal variation by the in *vitro* condition. Rice is mainly taken out for its high starch content having greater

food value. As it derived from the current experiment that in *vitro* ones showed a higher amount of chlorophyll as well as starch content in respect to in *vivo* ones thus enhancement of starch easily can be done in future through somaclonal variation by keeping them within culture condition for several periods. Probably by using this, after 3-4 cycle a high content starch yielding as well as protein and phenolics rich resistant variety will occur.

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