New aspects in rice tissue culture

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Abstract: In the last 10 years, micropropagation of cultivated plants has shown a picturesque development. Because of the importance of rice production in the world, extensive research efforts and progress have been made to improve yields. *In vitro* production efficiency of rice plantlets depends to the type of explants and *in vitro* abiotic factors. Different type of abiotic stresses including salinity, drought, low temperature, heavy metal and UV radiation, negatively influence the crop yield. To understand the stress induced plant responses and regenerate stress tolerant plantlets, environmentally controlled *in vitro* cultures are most remarkable.

Introduction: *Oryza sativa* L. includes in the family Poaceae and acts as the staple food of the world population. It is the main sources of carbohydrate, Thiamine, Riboflavin, Niacin and dietary fibre for human consumption (Heineman et al., 2005). The American Diabetes Association noted that carbohydrates are the body's main source of energy. The Glycemic Index of rice ranges from a low of 48 to a high of 92, with an average of 64 and the GI of rice depends on the type of rice consumed. More than 90% of rice is cultivated and consumed in Asia. The species contains two major sub-species, japonica and indica (Mackill, 1995; Aldemita & Hodges, 1996). The basic difference between these two ecotypes can be clearly recognized according to the distinct shape of the seeds. The ecotype, '*Indica'* is characteristically long and slim whereas '*japonica'* appears short and round. Rice occurs in different colours including white, red, black, brown and purple. Rice cultivation commonly done in temperate and sub-tropical climate under humid conditions. The abiotic factors like high humidity, temperature and rainfall are essential for better paddy cultivation. The plant tolerates both alkaline and acidic soils. The silts, loams and gravels are better soil types for rice growth (Khush, 1997).

The healthy rice seeds show better germination rate, while the germination rate of most rice seed begins to deteriorate rapidly after 6 months. The rice seeds are quite short-lived compared with seeds of other major crops, so they are often produced and stored under warm to avoid the loss of viability (Murata et al., 1968). The study on genes which responsible for high seed longevity opens up the production of rice seeds with improved longevity and vigor that can improve the livelihood of rice farmers. Probably, the adverse environmental conditions seriously threaten rice production. Both abiotic (drought, high salinity, high or low temperatures, flooding, high light, ozone, low nutrient availability, mineral deficiency, heavy metals, pollutants, wind and mechanical injury) and biotic (viruses, bacteria, fungi, nematodes, insects and herbivores) stresses often prevent the achievements of optimum rice yields (Wood et al., 2012).

***In vitro* rice culture:** Micropropagation or tissue culture is the technique of producing selected plants of known desirable agriculture qualities in large numbers from small explants in relatively short period of time. The term tissue culture refers to all type of cultures including meristem culture, embryo culture, ovule culture, anther culture etc (Debergh & Read, 1991). It is a method of rapid plant propagation under controlled disease free condition. Entire crop population with premium qualities can be created from a single elite specimen plant. Depending on the species, the original explant may be taken from shoot tip, leaf, lateral bud, seed, stem or root of the mother plant. Explants from selected mother plant are established and multiplied under *in-vitro* conditions by providing the optimum pre-requisite for plant growth. These explants go through the initiation, multiplication and rooting for producing a cell into a full-fledged plant. This ability of a single cell to divide, multiply and produce a new differentiated plant is referred to as totipotency (Vasil & Vasil, 1972). The tissue culture is now widely applied to regenerate commercially important plant species including *Cocos nucifera* L., *Phoenix dactylifera* L., *Anacardium occidentale* L., *Mangifera indica* L., *Citrus reticulata* Blanco etc (Silva & souza, 1992; Hornung, 1995; Mukhopadhyay, 1997; Al-Khayri, 2007; Mishra, 2010). *In vitro* propagation in *Phalaenopsis sp.*, *Dianthus caryophyllus* L., *Gladiolus anatolicus* (Boiss.) Stapf, *Gerbera jamesonii* Adlam, *Anthurium andreanum* Linden etc. are also important in horticultural field (Jain et al., 1997; Kosir et al., 2004; Emek & Erdag, 2007; Bhatia et al., 2009; Celik & Atak, 2009).

Rice plant regeneration from shoot tips is rare in tissue culture studies. While the use of seeds as explants in japonica varieties are most investigated. Seed embryo in rice is better explant in genetic transformation studies. Availability and easy to handling of seeds makes the micropropagation most successful. The callus induction and somatic embryogenesis in indica varieties are hardly reported due to the recalcitrant nature. Callus is the mass of undifferentiated tissue formed by the dedifferentiation of the explants. From each immature cell, the single plantlets are regenerating on artificial culture medium supplemented with different concentrations of auxins and cytokinins. From each somatic cell of callus, single embryos are formed that in turn form new plantlets, the entire process is known as somatic embryogenesis (Ilahi et al., 2005). In case of somatic embryo, there is no endosperm and seed coat.

Based on the plant or explant selected, we have to choose the suitable media. The culture media used for plant tissue culture may be liquid or semi-solid in nature. The commonly used media included Gamborg medium, White's medium, Murashige and Skoog medium, N6 medium, B5 medium etc (Street, 1973). Murashige and Skoog medium (MS) is an artificial plant growth medium commonly used in the tissue laboratories for culturing of plant tissues. It was invented by Toshio Murashige and Folke K. Skoog in 1962 during the study on plant growth regulator controlled *in vitro* plant regeneration. This medium consisted of micronutrients, macronutrients, vitamins and organic compounds which are essential for the normal growth and development of plants. It has balanced nutrient composition over other media, so it is highly suitable for majority of the plant species culture (Murashige & Skoog, 1962).

For *in vitro* rice regeneration, the most suitable medium selected by researchers is MS medium. The 2,4-D (2,4-dichlorophenoxyacetic acid) is a synthetic auxin and is the most commonly used growth regulator in culture of cereals (Ozawa & Komamine, 1989; Naqvi et al., 2002). By the supplementation of 2,4-D in MS medium induces callus from the rice seed embryo of Govind, Pusa Basmati-1, Basmati-370, Basmati-385, Jaya, Navara, Sita, Rupali, Swarna Masuri etc. For callus proliferation 2,4-D and Kinetin or BAP are also supplementing with MS medium. The rice calli are creamy white in colour, friable and embryogenic. Somatic embryogenesis and organogenesis are triggered by auxins and cytokinins. However, BAP together with Kinetin induces somatic embryos from immature tissues of calli. The shoot regenerates by the applications of BAP, Kin and NAA at different concentrations in MS medium (Roy & Mandal,

2006). For root regeneration, the auxins like NAA and IBA are supplying to the plantlets which cultured on the MS medium. The *in vitro* plants are hardening in sterilized soil after proper growth and development.

Hardening may be primary or secondary in process, of which primary hardening included incubation of *in vitro* plantlets in plastic pots contained the liquid nutrient medium for 6-8 weeks in green house. Then these hardened plants transfer in to polythene bags contained the potting mixture and keep in shade house for 6-8 weeks (Chandra et al., 2010). After this secondary hardening, the plants transfer in to the cultivation field. The step wise hardening process helps to adjust the rapid abnormal environmental conditions by irradiance, temperature, air flow, moister etc. This acclimatization will overcome the threat due to the adverse environmental conditions by controlling both physical and chemical environment (Hazarika, 2003).

Fig 1. A. Seeds of *Oryza sativa* cv. Navara, B. Seed germination on MS medium, C. Callus induction on MS+2,4-D1mg/l, D. Shoot induction on MS+BAP1.0mg/l+NAA0.5mg/l, E. *In vitro* Navara seedlings.

Rice is a single seeded fruit called caryopsis. It has an outer protective covering, the pericarp, seed coat, nucellus, aleurone layer, embryo and endosperm. Rice seeds show a dormancy period immediately after harvest. The loss of viability of seeds within one year after harvesting has been reported by Grist from varieties of rice. To overcome this viability loss, micropropagation is a better method to produce a lot of progenies from a single seed embryo that cause genetic stability and germ plasm conservation. The explants selected for *in vitro* culture are with superior quality. From a single explant, we can produce a number of similar rice progenies with superior quality.

***In vitro* rice culture under salinity stress**: Presence of excess salinity in soil is one of the major abiotic factors that limit plant growth and development of a wide variety of crops including rice. Salinity can be considered as severe abiotic stress which includes all the problems due to salts primarily by an abundance of sodium chloride from natural accumulation or irrigation practices (Flowers & Flowers, 2005). Plants can be mainly divided into two groups based on the effect of salt on plant development, glycophytes and halophytes. Glycophytes are very sensitive to soil salinity, whereas halophytes can tolerate high salt concentrations (Tuteja et al., 2011). Salinity stress results in growth reduction, stomatal closure, leaf senescence, loss of seed viability and deterioration of crop yield.

On the basis of tolerance potential toward salinity stress, rice is considered as salt-sensitive crop that in turn influence the growth and yield (Momayezi et al., 2009). While some of the rice varieties are salt tolerant. Rice plants generally tolerate salt stress by mainly two mechanisms, ion exclusion and osmotic tolerance that prevent the accumulation of toxic concentrations of Na^+ and Cl^- in plant tissues (Munns & Tester, 2008). Some of the important class of salt tolerant genes in rice plants are *OsSOS1*, *OsNHX1* (Na^+/H^+ antiporters) (Kumar et al., 2013, Amin et al., 2016), *OsHKT2;1* (Na^+/K^+ symporter) (Mishra et al., 2016), *OsCAX1* (H^+/Ca^+ antiporter) (Kumar et al., 2013), *OsAKT1* (K^+ inward- rectifying channel) (Yang et al., 2014), *OsKCO1* (K^+ outward-rectifying channel) (Kumar et al., 2013), *OsTPC1* (Ca^{2+} permeable channel) (Kurusu et al., 2012), *OsCLC1* (Cl^- channel) (Diedhiou & Golldack, 2006) and *OsNRT1;2* (nitrate transporter) (Wang et al., 2016) that code stress tolerant proteins.

Salinity threat is estimated to reduce global rice production by 50%. Rice is susceptible to salinity specifically at the early vegetative and later reproductive stages. To evaluate the effect of different concentrations of NaCl on *in vitro* culture, many researchers selected separate varieties

and identified the salt tolerant potential. Callus from seed embryo of different varieties is normally induced on MS medium fortified with 2,4-D at specific concentration. This callus is sub-cultured on MS medium with 2,4-D and different concentrations of NaCl. In PAU 201, PR116, Pusa Basmati 1, Basmati 370, Type III, Pant Dhan 4, CSR 10, Navara and Pokkali, the callus fresh weight is decreased as concentration of NaCl increased from 0 to 150mM (Shankhdhar et al., 2000; Wani et al., 2010; Hima & Deepa, 2018). Moreover, these varieties consider as salt tolerant varieties due to the survival of calli at particular NaCl concentration. The normal callus proliferation shows in cultures on MS medium without NaCl. When the calli transfer from low to high salt medium, it is immediately subjected to osmotic dehydration. NaCl acts as the ionic and penetrating stress agent and induces the toxic salt injuries in callus. This toxicity gradually changes the colour of callus from pure white to yellowish brown due to osmotic dehydration.

Drought tolerance in rice: Seasonal drought is a major threat to rice cultivation. Rice is highly susceptible to drought during the reproductive stage, leading to significant reduction in grain quality. Drought stress adversely influences on cell growth, cell elongation and cell expansion that disrupts plant antioxidant function by promoting reactive oxygen species accumulation (Singh et al., 2012). To overcome the present issue, the regeneration of drought resistant seedlings is more significant. Drought resistance is an important quantitative trait that controlled by more than one gene. Drought stress responses can be managed by adopting different strategies including breeding and marker assistant selection, modulation of drought responses through the application of plant hormones, enhancement of osmotic adjustment with osmolytes, improvement of antioxidant functions as well as generation of transgenic drought tolerant rice plants (Upadhyaya & Panda, 2019). Presently it is identified that WRKY gene family members are playing significant role in drought tolerance. A total of 89 WRKY genes in *japonica* and 97 WRKY genes in *O. nivara* (OnWRKY) have been identified and mapped onto individual chromosomes (Sahebi et al., 2018).

Fig 2. A. Callus proliferation on MS+2,4-D1mg/l+60mMNaCl, B. Shoot induction on MS+BAP0.5mg/l+IBA1.0mg/l+60mMNaCl, C. *In vitro* seedling regeneration on MS+BAP0.5mg/l+IBA1.0mg/l+60mM NaCl.

By using polyethylene glycol (PEG) as drought stress inducing agent, many *in vitro* studies in different rice varieties were carried out by researchers that including IR 18351-229-3, IR 3185-6-3-3-2, SR 26-B, Nona Bokra, PAU 201, PR 116 and C 14-8. Callus induces normally from mature seeds cultured on MS medium supplemented with 2,4-D and PEG at different concentrations. These varieties show differential drought resistance under *in vitro* conditions of which SR 26-B and C 14-8 never show drought stress symptoms during seed germination, callus induction and plant regeneration. These varieties can be used as drought tolerant lines. While a gradual reduction in *in vitro* responses exhibits by IR 18351-229-3, IR 3185-6-3-3-2 and Nona Bokra in presence of increased concentrations of PEG (Biswas et al., 2002; Wani et al., 2010).

The transcription factor, OsLG3 positively regulates drought tolerance in rice. The overexpression significantly improves the drought tolerance of rice plants, whereas the suppression of OsLG3 results in drought susceptibility. It strongly expresses in upland rice than in lowland rice under drought stress conditions. This transcription factor has a positive effect on

rice grain length without affecting grain quality (Xiong et al., 2018). The drought tolerant rice lines can be produce by inserting this corresponding gene using *Agrobacterium* mediated transformations. This genetically engineered plant will encode the proteins involved in drought stress regulatory networks by the translation of specific genes (Kumar, 2019). Drought-inducible receptor-like cytoplasmic kinase, named Growth Under Drought Kinase (GUDK), is essential for better grain yield under drought and well-watered conditions. GUDK mutant lines exhibit sensitivity to salinity at the seedling stage and a reduction in photosynthesis under controlled drought stress at the vegetative stage (Ramegowda et al., 2014). The dehydration response element binding factors (DREB) also plays a major role in response to drought stress in rice. Two indica varieties, Sambha mahsuri and Cotton dora sannalu have been transformed for the gene AtDREB1A under 35s CaMV promoters (pBIH binary vector) for which the vector used was *Agrobacterium* (Reddy et al., 2018).

UV induced stress responses in rice: Stratospheric ozone depletion leads to a significant enhancement in UV radiation reaching the surface of the Earth (Soheila, 2000). This elevated UV is potentially deleterious to all living organisms. Normally, the plants have protective mechanisms against UV stress, such as enhancement of the antioxidant system and accumulation of UV-absorbing compounds (Brosche & Strid, 2003; Frohnmeyer & Staiger, 2003). UV radiation induces the generation of signal transduction intermediates such as nitric oxide, reactive oxygen species (ROS) and ethylene. It down-regulates the photosynthesis-related proteins such as light-harvesting Chl *a*/*b*-binding protein and up-regulates protective proteins such as pathogen-related protein-1 (PR-1), pigment-dispersing factor 1.2 (PDF 1.2) and expression of senescence-associated genes at the mRNA level (Jordan, 2002; Kalbina & Strid, 2006).

The UV augmentation causes significant variation in vegetative growth in Sasanishiki, Norin 1 and Surjamkhi in turn influences the reproductive stages (Hidema et al., 2007). UV-B radiation sharply decreases the content of Rubisco in Surjamkhi and has no effect in Sasanishiki. The photochemical activities of photosystem (PS) 1 and PS 2 slightly affect by UV-B radiation. Moreover, the activities of antioxidant enzymes, catalase (CAT), peroxides (POX) and superoxide dismutase (SOD) enhance by UV-B stress. Among the three varieties, CAT and POX activities are enhancing in Sasanishiki by UV-B radiation than those in Norin 1 and

Surjamkhi (Fedina et al., 2010). Norin 1 photolyase dimer complex was highly thermolabile relative to the wild-type Sasanishiki photolyase. The deficiency in photorepair of cyclobutane pyrimidine dimers results from a structural/functional alteration of photolyase. Thus, the molecular origin of this plant DNA repair deficiency, resulting from a spontaneously occurring mutation to UV radiation sensitivity, is by defective photolyase (Hidema et al., 2000). Some researchers are trying to induce UV stress in *in vitro* rice culture, but more information is not published yet.

Low temperature stress in rice: When the plants of tropical and subtropical origin exposed to non-freezing temperatures below 10-15^{0}C, there occurs some chilling injuries in plant tissues (Saltveit, 2000). Cold stress can be classified as chilling (0-15°C) and freezing (<0°C) stress, which is a major environmental factor limiting the growth, productivity and geographical distribution of crops (Zhu et al., 2007). Due to its origin in tropical and subtropical regions, rice is more sensitive to cold stress than other cereal crops such as wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Xie et al., 2012). Low temperature strongly suppresses many proteins and genes of rice seedlings, but also induces several functional genes and proteins (Minhas & Grover, 1999; Sung et al., 2003). These proteins act as the adaptation mechanism to low temperature stress conditions (Thomashow, 1999; Sung et al., 2003). In rice seedlings, low temperature rapidly induces gene expression of alcohol dehydrogenase (ADH) (Christie et al., 1991; Minhas & Grover, 1999).

Dissecting cold stress-mediated physiological changes and understanding their genetic causes will facilitate the breeding of rice for cold tolerance. Cold stress affects chlorophyll content and fluorescence that interfere with photosynthesis in rice (Kim et al., 2010). The increased contents of reactive oxygen species (ROS) and malondialdehyde (MDA) that accumulate during cold stress can impair metabolism via cellular oxidative damage (Xie et al., 2009). On the other hand, rice also possesses strategies to cope with or adapt to cold stress. The cold-treated rice plants accumulate proline that stabilizes protein synthesis and maintains the optimal function of rice cells (Kandpal & Rao, 1985).

An effective way to breed cold-tolerant cultivars is the usage of molecular markers and linkage maps. Many cold-tolerance related QTL in rice have been identified in the past 20 years. The QTL *Ctb1, qCTB2a, qPSST-3, qLTB3* are related to cold tolerance at the reproductive

stage; *qCTP11* is related to cold tolerance at the germination stage; and *qCtss11* and *qCTS4a* are related to cold tolerance at the seedling stage. The identification of cold tolerance-related QTL has more significance presently, because of urgent need of cold-stress resistant rice seedlings (Zhang et al., 2014). The cold tolerant and sensitive genotypes, Nipponbare and M202, show a clear difference in gene expression at the transcript level for *OsGH3-2*, *OsSRO1a*, *OsZFP245* and *OsTPP1* as well as for expression at the protein level for LRR-RLKs, bHLH, GLYI and LTP1 proteins (Freitas et al., 2019).

Heavy metal stress in rice: The antropogenic activities enhance the concentration of hazardous chemicals in the environment that continuously enter to aquatic and terrestrial ecosystems. To remove this harmful chemical compounds from nature, the use of green plants are common and this process is phytoremediation (Cunningham et al., 1996). The natural plants or genetically engineered plants can be utilized to extract the radioactive or toxic organic compounds from soil.

Heavy metals contamination in paddy field is a common phenomenon that observed in last few years. We apply agrochemicals especially chemical fertilizers in paddy fields, which release potential toxic heavy metals into soil. The rice plants absorb the toxic ions and accumulate in different plant parts including grains. The rice plants are generally contaminated by many heavy metals including lead, cadmium, chromium, copper, manganese, arsenic and zinc (Wang et al., 2006; Payus & Talip, 2014). The ranking order of bioaccumulation factor (BAF) for heavy metals is Zn > Mn > Cd > Cu > Cr > Pb, indicating that the accumulation of micronutrients is more than that of nonessential toxic heavy metals in rice (Satpathy et al., 2014). Normally the heavy metal accumulation causes reduction of growth, carbohydrate content and net photosynthesis in rice plants. While the applications of gibberellins (GA_3) and abscisic acid (ABA) in rice will partially reverse the effect of heavy metals and stimulate growth as well as mobilization of carbohydrate in seeds from which seedlings had developed (Moya et al., 1995).

The mechanisms to minimize the concentrations of heavy metals in rice grains are important. It is possible by water management including continuously flooding and alternate wetting and drying (AWD) methods. The continuously flooded cultivation reduces the concentration of heavy metals in the rice grains by reducing the root-to-shoot translocation and the availability of metals in rhizosphere. The flooding significantly decreased the iron plaque on the root surface

and reduced the affinity for metals in rhizosphere. The increasing of abundance of unique bacterial community also reduces iron plaque formation and metal affinity in rhizosphere that diminish the uptake and accumulation of heavy metals in rice plants (Zhang et al., 2019).

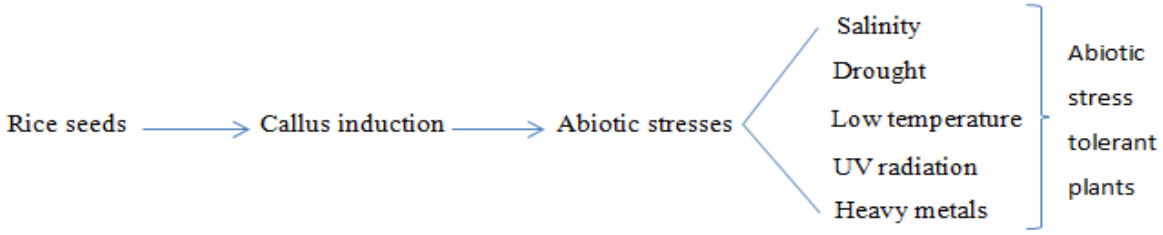

Fig 3. *In vitro* regeneration of abiotic stress tolerant plants from callus.

Secondary metabolite production in plants under abiotic stresses: In plants, there are two types of metabolism, primary and secondary metabolism. Primary metabolism is included the production of metabolites such as carbohydrates, amino acids, lipids etc., that utilized by the plants for their normal growth and development. On other hand secondary metabolism is associated with the production of compounds involved in the protection of plants against various abiotic and biotic stresses. Under stresses, the plants can synthesize several secondary metabolites to cope with the adverse effects of stresses.

When plants recognized the stress at the cellular level, a particular stress response is induced to overcome the adverse condition. During drought, the plants tend to decrease the leaf area to reduce the water loss through respiration that promotes the leaf abscission. It is regulated by the effect of specific plant hormones. When the plant suffers with oxidative stress, they synthesize the various secondary compounds and increase the level of different endogenous enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR). The non-enzymatic antioxidants like ascorbate (AsA) and glutathione (GSH) also produced to reduce the effect of reactive oxygen species (ROS). The various secondary metabolites produced in plants during abiotic stresses include flavonoids, anthocyanins, phenylpropenoids, phenolic compounds, monoterpenes, essential oils, morphin, sorbitol, polyamines, agmatine, putrescine, quercetin, kaempferol and carotenoids (Kumar & Sharma, 2018).

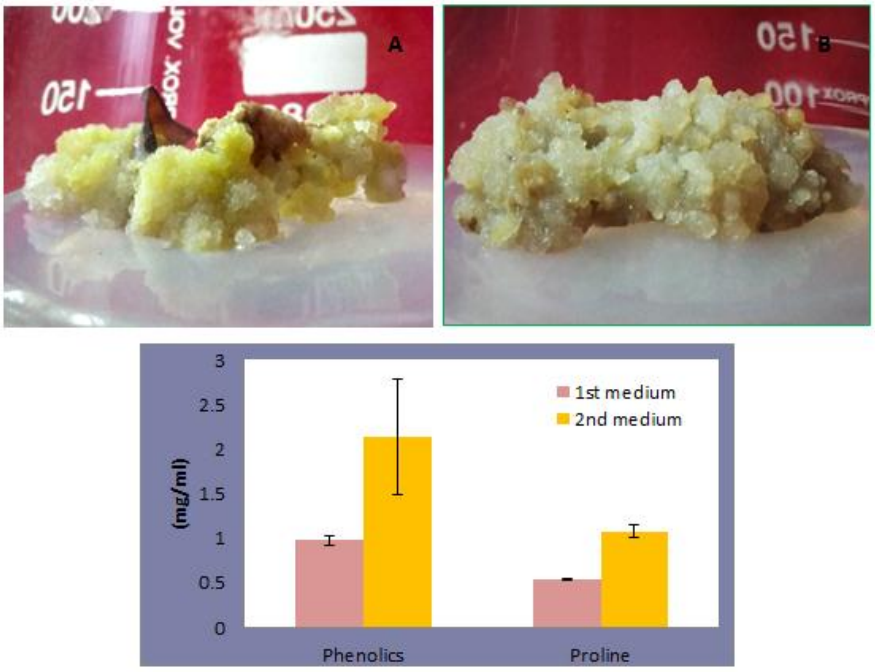

Fig 4. A. Callus induction from leaf of *Centella asiatica* (L.) Urban on MS+2,4-D1mg/l+ KIN0.5mg/l+TRY4mg/l, B. Callus proliferation on MS+KIN0.5mg/l+BAP0.3mg/l +40mMNaCl, C. Phenolics and proline quantified from calli grown on MS medium supplemented with KIN0.5mg/l+BAP0.3mg/l+40mMNaCl (2^{nd} medium) and KIN0.5mg/l+ BAP0.3mg/l (1^{st} medium).

Conclusion: Tissue culture is the more convenient and cost effective method for producing stress resistant plants. The technique is under the controlled environmental conditions and need less time and space. This method can be used as a better strategy to understand the biochemical, physiological and molecular changes in *in vitro* plants under abiotic stress. *In vitro* studies also help to detect the phytoremediation potential of different plant species. These plants can be applied in heavy metals contaminated soil which in turn nullifies the toxicity of soil.

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