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Development of an *in vitro* plant regeneration protocol in the spotted duckweed, *Landoltia punctata*.

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Abstract: ***Landoltia punctata*** **is an aquatic, free-floating angiosperm plant with a very high multiplication rate and numerous commercial uses.** ***In vitro*** **plant regeneration protocols are necessary to exploit these plants as biomanufacturing platforms. It is frequently observed that the protocols established elsewhere are not effective when it was adopted by another group of researchers due to various reasons. In an attempt to develop transgenic plants in** ***L. punctata*****., the present study developed a successful and reproducible plant regeneration protocol using fronds as explants. Gamborgs's B5 media and MS media were used in the protocol. Callus induction was obtained on Gamborg's B5 media supplemented with BAP (1 µM), Dicamba (20 µM), and sucrose (2%). For callus growth MS medium with 2, 4-D (30 µM), TDZ (1 µM), and sorbitol (2%) were used. MS medium supplemented with TDZ (15 µM) and the carbon source combination of sorbitol and sucrose (2:1) gave efficient plant regeneration in** ***L. punctata*****. Half-strength basal MS media was used for rooting and the in vitro regenerated plants were successfully transferred to the maintenance solution (half-strength Hutner's media) within a span of one week. The established protocol will be used to develop transgenic L. punctata plants expressing recombinant proteins.**

Index terms: callus growth, frond regeneration, ***in vitro*** **propagation,** ***L. punctata***

I.INTRODUCTION

Duckweeds are a small group of free-floating aquatic flowering plants belonging to the family Lemnaceae, distributed in freshwater lentic ecosystems of tropical and temperate regions (Landolt, 1986). Taxonomic studies using various molecular tools reveal that Lemnaceae consists of 5 genera and 36 sp. (Bog et al., 2019). Out of these five-genera, three genera including *Spirodela*, *Landoltia*, and *Lemna* produce both fronds and roots, and the other two genera, *Wolffiella*, and *Wolffia* produce only fronds (Landolt, 1986). Duckweeds reproduce mainly through asexual methods. Nutrients, photoperiod, and hormones play a major role in flowering, and the lentic habitat may be providing the minimum variation leading to a dominant asexual mode of reproduction as flowering is very rare in the Lemnaceae family (Stomp 2005; Demirezen et al., 2007; Fourounain et al., 2021). The neotenous lifestyle of the family makes them the fastest-growing angiosperms with an approximate doubling time of 24h to 48h (Stomp 2005; Burns et al., 2015). Studies on the chemical composition of different duckweed species propose it as a potential future food (Appenroth et al., 2017; Yahaya et al., 2022; Xu et al., 2021). Duckweeds (especially *Wolffia*) have been used extensively as food in East Asian countries like Thailand and Vietnam (Saengthongpinit, 2017). Other uses of duckweeds include, as fodder for livestock (Lasocinki., 1979; Chojnacka., 2006; Gupta and Prakash., 2013), as a source of protein (Gupta and Prakash., 2013), as a constituent of phytocosmetics (Czerpak and Piotrowska, 2005), and biopharmaceuticals (Vladimirova and Georgiyants., 2014; Karamalakova et al., 2022). The rapid multiplication nature of these plants makes them an excellent candidate to be used in environmental reclamation programs including the generation of biomass for green energy (Verma and Suthar., 2014; Romanwaska-Duda and Pszczolkowski., 2013; Zhao et al., 2014; Cui and Cheng., 2015; Evins et al., 2019), and in phytoremediation (Al-Khafaji et al., 2018; Bell et al., 2003; Bonomo et al.,1997; Dalu et al., 2003). Exponential growth and simple growth requirements make the duckweed family an inevitable group in molecular farming for recombinant proteins (Yang et al., 2021; Ko et al., 2011). Stable transformation of duckweeds has been reported in *Lemna* (Canto pastor et al., 2015;

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Nguyen et al., 2012; Firsov et al., 2018; Firsov et al., 2015; Yang et al., 2018; Ko et al., 2011; Liu et al., 2019; Moon et al., 1998; Stomp and Rajbhandari, 2000; Yamamoto et al., 2001) *Spirodela* (Balaji et al., 2015, Peterson et al., 2021), *Landoltia* (Wang et al., 2021; Rival et al., 2008, Vunsh et al., 2007), and *Wolffia* (Bohem et al., 2001; Kruse et al., 2002; Friedrich et al., 2005). Cox and co-workers have engineered the protein glycosylation pathway in *Lemna minor* for expressing humanized monoclonal antibodies against human CD30 using RNA interference (Cox et al., 2006). Biologically active antibodies compatible with the mammalian system expressed in *L. minor* (Bertran et al., 2015; Gasdaska et al., 2011), indicating the potential of duckweeds as a therapeutic protein expression platform.

Establishment of an *in vitro* plant regeneration system in Lemnaceae members is important due to its special features and commercial potential for various application. While there are few successful reports on the *in vitro* plant regeneration and transformation, the established protocols seem to be non-responsive when the plants are collected from a different location. This makes it necessary to standardize the plant regeneration procedure every time we collect the explant from a new location. *L. gibba* were the first group of Lemnaceae used in the *in vitro* plant regeneration studies around four decades ago Change and Chiu (1976, 1978). Since then, there have been a continuous effort to improve the plant regeneration protocol of duckweeds due to its multifaceted application potential. Being a member of the monocot family, *in vitro* cell multiplication and regeneration of Lemnaceae is time taking and needs continuous subculture in different, nutrient and phytohormone combination (Chang and Chiu, 1976; Khvatkov et al., 2015; Stefaniak et al., 2002). Callus induction and plant regeneration have been reported in a few Lemnaceae species, including *L. punctata* (Li et al., 2004), *S. polyrhiza* (Wang et al., 2010) *S. punctata* (Frick et al., 1991; Decock et al., 1979), *L. gibba* (Moon and Stomp, 1997), *L. minor* (Chhabra et al., 2011), *Wolfia arrhiza* (Khatkov et al., 2015). The differences in response observed by various groups worked on *in vitro* plant regeneration endorse that the *in vitro* response of different ecotype of the same species vary greatly (Moon and Stomp, 1997). The major determinants of callus induction and plant regeneration include the type of explant, composition and pH of the medium, plant growth regulators, light, and temperature (Moon et al., 1998; Moon and Stomp, 1997).

Spirodela punctata (dotted duckweeds) (renamed as *Landoltia punctata* (Les and Crawford 1999)) is one of the most prevalent duckweed species worldwide with a very high growth rate (up to 39.2 to 44 t dw/ha-year). It has been used widely by people around the world, mainly as food and fodder. High protein turnover (more than 40% under optimum condition) indicates its potentials to be exploited as an *in vitro* protein expression platform. In the present study, an *in vitro* plant regeneration protocol has been developed for *L. punctata* plants in order to develop transgenics for recombinant protein expression. Different media, phytohormones, and culture conditions were used to establish a successful *in vitro* plant regeneration protocol. The experiments were conducted using different media formulation, carbon sources, and phytohormone combination. The study established a successful *in vitro* plant regeneration protocol using fronds as the explant within a span of two months.

II. RESULTS

A. CALLUS INDUCTION

Callus induction was observed when the fronds cultured on Gamborg's B5 medium supplemented with the cytokinin (BAP/TDZ) 1µM and varying concentrations of auxins DIC and 2, 4-D (0-50µM). The carbon supplement used were sucrose and sorbitol (20g/L). A higher rate of callus induction (>90%) was observed with the media having 1µM BAP, 20µM DIC, and 2 % sucrose.15 and 25µM DIC also gave callus induction responses at a rate of 80 and 85% respectively. While sucrose found to be the carbon source facilitating the callus induction with a range of auxin concentrations, the response of explants with sorbitol was significantly less (< 50%) (Fig-1). The TDZ/2,4-D combination with sorbitol found to be less responsive while sucrose maintained a high degree of response with the same combination. Even though the medium supplemented with 2% sorbitol and BAP/DIC combination was giving the callus induction considerably good (~50%), it was less than the sucrose containing medium with the same phytohormones (Fig- 4 c). Calli induced with sucrose + BAP/DIC were selected for the callus growth experiments.

B. CALLUS GROWTH

Development of an undifferentiated mass of cells after two weeks of incubation period was attained using MS medium supplemented with a combination of BAP/ TDZ and 2,4-D. While sucrose was giving the callus induction responses with the fronds, sorbitol was effective in giving significant improvement in callus growth. A combination of TDZ (1µM) and 2,4-D (30µM) found to give the maximum callus growth (91.66%) in *Landoltia*. When sorbitol was substituted with mannitol, the competency of callus growth decreased up to 40% and the calli were non-responsive after one weak of incubation (Fig-2). Callus growth response was minimal in mannitol (<50%) with both TDZ and BAP. Among the cytokinins, TDZ gave better response with sorbitol than with BAP (90% against 75%). The calli developed on sorbitol with the combinations of BAP/TDZ with 2,4-D were taken for the plant regeneration experiments. For long term callus maintenance, a combination of 1µM TDZ, 30µM 2,4-D and 2% sorbitol, were used (Fig- 4 d).

C. FROND REGENERATION

Frond regeneration was attained with 3-4 weeks old callus when transferred to MS media containing the

cytokinin TDZ or BAP in various concentrations. Among the different carbon sources tried, a combination of sorbitol and sucrose (2:1) responded better than the carbon sources used alone. 3% sucrose with BAP also gave the frond regeneration in *Landoltia* (55%). The phytohormone TDZ (15µM) with the combination of carbon source gave the highest percentage of frond regeneration (93.33%) (Fig-3). Successful frond regeneration was observed after 2-3 weeks of incubation (Fig- 4 e and f).

D. ROOTING AND TRANSFER TO LIQUID MEDIUM

Regenerated fronds were transferred to half strength MS basal media for root regeneration. Fully developed roots were formed within 7-10 days on the solid media and the plants were transferred to half-strength Hutner's liquid media. The plants started multiplying in the media and attained the normal multiplication frequency within a week (Fig- 4 g, h and i).

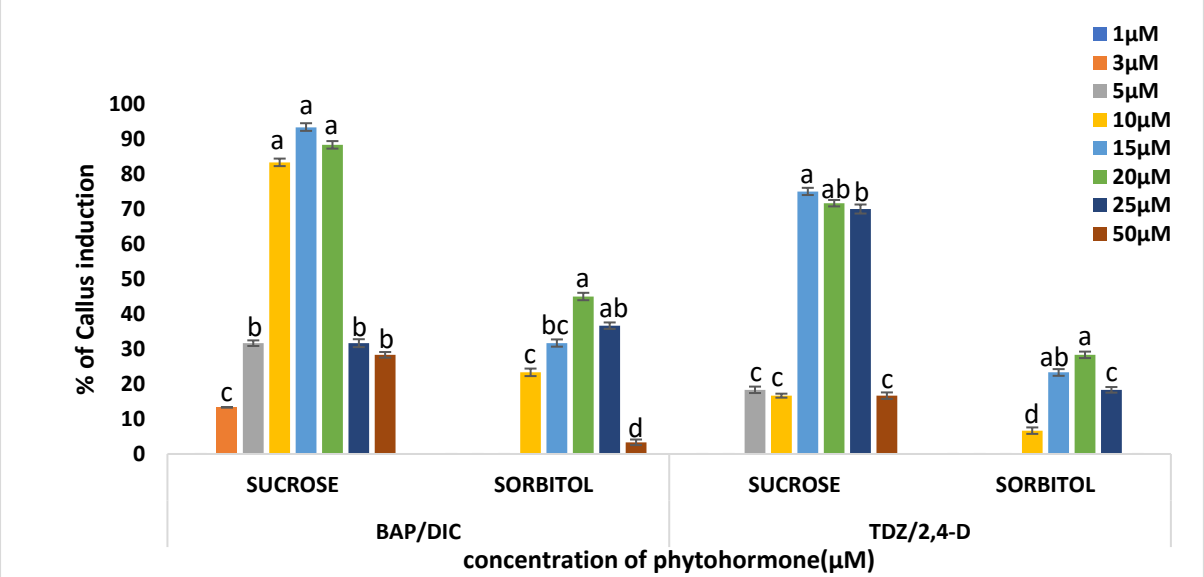

Fig 1. Callus induction in *L. punctuata*. Effect of plant growth regulators and carbon source on callus induction in *L. punctata*. Gamborg's B5 media supplemented with different phytohormones/carbon source (x-axis) showed callus induction with the frond explants and the response percentage is given in the y-axis. BAP- 6-Benzylaminopurine, TDZ- Thidiazuron, DIC - Dicamba, 2,4-D - 2,4- Dichlorophenoxyacetic acid. All the combinations were used in triplicates with a minimum of 20 explants each. All data were showing significant variation having $p \leq 0.05$ using one-way anova test. All graphical data analysis are presented as mean ± standard error.

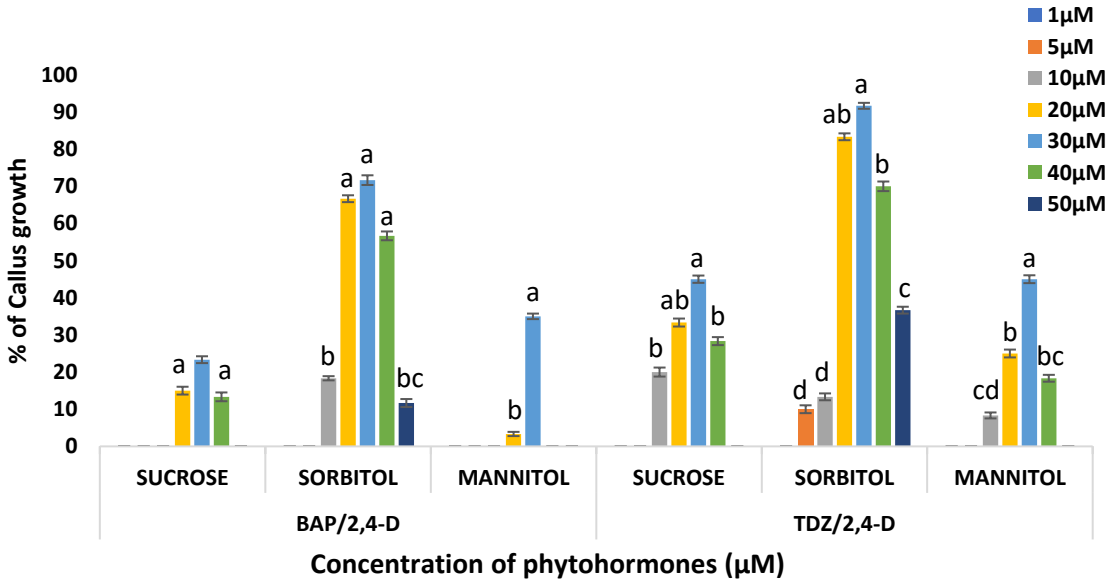

Fig 2. Callus growth in *L. punctuata*. Effect of plant growth regulator and carbon source on callus growth in *L. punctata.* MS media supplemented with different phytohormones and carbon sources (x-axis) and the response percentages (y-axis) are given. BAP- 6-Benzylaminopurine, TDZ- Thidiazuron, 2,4-D - 2,4- Dichlorophenoxyacetic acid. All the combinations were used in triplicates with a minimum of 20 calli each. All data were showing significant variation having $p \leq 0.05$ using one-way anova test. All graphical data analysis are presented as mean ± standard error.

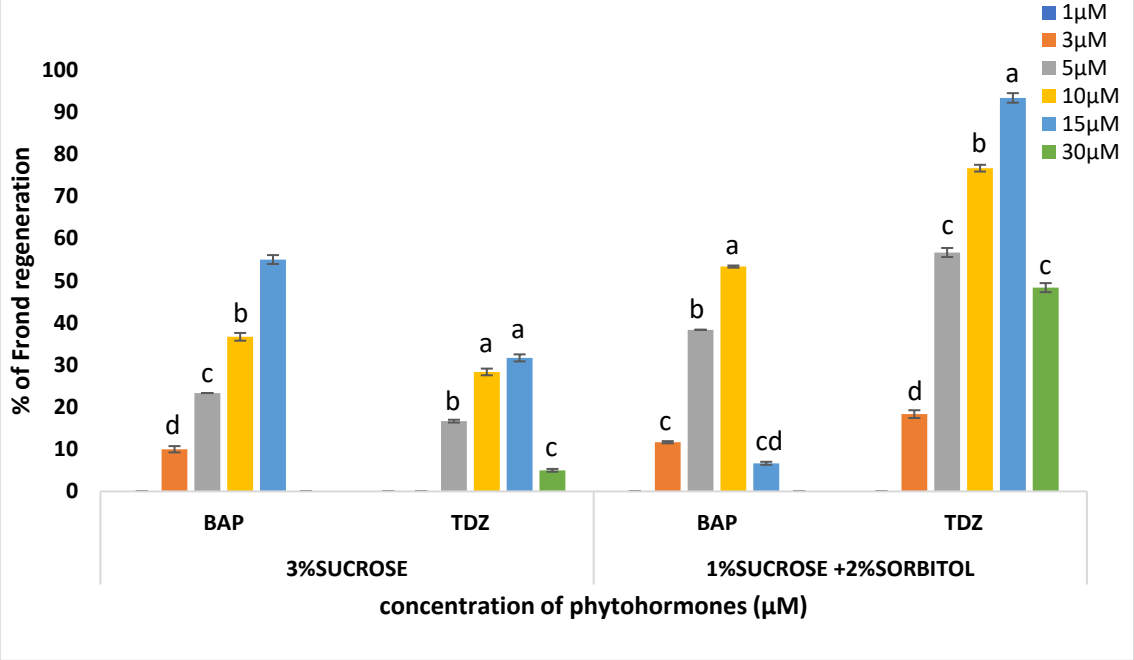

Fig 3. Frond regeneration in *L. punctuata*. Effect of BAP/TDZ and carbon sources on frond regeneration in *L. punctata.* MS media supplemented with BAP/TDZ with different carbon source used in the frond regeneration of *L. punctata*. BAP- 6-Benzylaminopurine, TDZ- Thidiazuron. All the combinations were used in triplicates with a minimum of 20 calli each. All data were showing significant variation having $p \leq 0.05$ using one-way anova test. All graphical data analysis are presented as mean ± standard error.

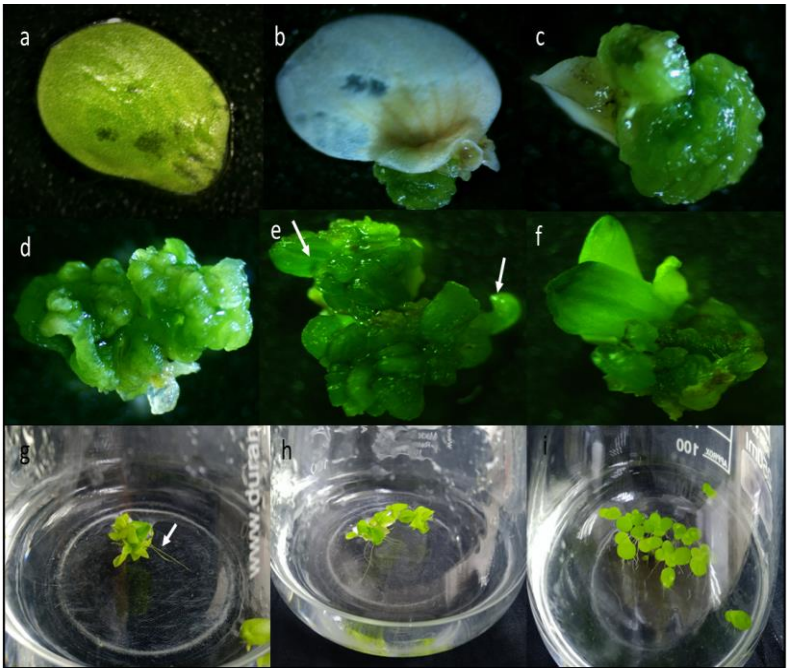

Fig 4. Different stages in the *in vitro* plant regeneration in *L. punctata*. (A) Mother fronds cultured on Gamborg's B5 medium containing 2% sucrose,1µM BAP and 20µM dicamba. The fronds get bleached and the meristematic regions start dividing to form the callus. The fronds were maintained for two weeks. **(B, C, D)** different stages of callus growth on MS media containing 2% sorbitol,1µM TDZ and 30µM 2,4-D. Calli grow to multiple patches of cells later form the fronds on

frond regeneration media. The callus after three to four weeks transferred to frond regeneration media. **(E)** Induction of frond regeneration on MS medium containing 1% sucrose+ 2% sorbitol and the phytohormone (TDZ 15µM) after 7-12 days of incubation. The arrows indicate the new fronds developed from the calli. **(F)** Green healthy regenerated frond after 14 days incubation period. **(G, H, I)** The regenerated frond shifted on half strength MS basal media developed roots (arrow) within 10 days and later transferred to Erlenmeyer flasks containing half strength Hutner's media.

III. DISCUSSION

In vitro plant regeneration in duckweeds is of immense importance due to the commercial exploitation potential of its members. So far, out of 38 species of duckweed only a few sp. Such as *S. polyrhiza S. oligorrhiza, L. punctata, L. gibba* var. Hurfeish, *L. minor*, and *W. arrhiza* have had an established and viable frond regeneration protocol. Considering the disparities existing among various published protocols on same species and the changes in the *in vitro* response due to the adaptations obtained under different environmental conditions, it become necessary to standardize the *in vitro* plant regeneration protocol whenever the plant is being chosen as a protein expression platform. The effectiveness of callus induction, callus growth, and frond regeneration depends on many factors including explants, medium composition, growth condition, sugar supplements, and plant growth regulators.

The callus induction from the fronds of *L. punctata* was obtained on Gamborg's B5 media supplemented with 2% sucrose, 1µM BAP and 20 µM DIC with a response percentage of 93.33% compared to other carbon sources, and phytohormones. In addition, when Gamborg's B5 media supplemented with 2% sorbitol, 1µM BAP, and 20µM 2,4-D was used, a notable decrease in callus induction efficiency i.e., 51.66% was observed. So, in our study, sucrose was found to be the best carbon source option for callus induction with *L. punctata*. TDZ and 2,4-D with 2% sucrose and 2% sorbitol also produced callus but at the rate of 33.33% and 35% respectively. Li et al. (2004) developed calli from the frond explants of S. *oligorrhiza* and *S. punctata*, with a slower rate and over a longer time period. In *L. punctata*, the callus induction was obtained on 1/2MS salts+ B5 vitamins supplemented with 1% sorbitol+ 3.5mg/L 2,4-D+ 50mg/L DIC+ 2mg/L 2iP. *Spirodela oligorrhiza* strain SP did not produce callus on B5 medium, but when WP medium supplemented with 50 mg/L DIC was used, an induction rate of 91–95 % was obtained (Li et al. 2004). The impact of carbon sources on the development of calli, varies amongst duckweed species (Khvatkov et al., 2015; Li et al., 2004). According to Moon and Stomp (1997), sucrose was found to be the best carbon source for *L. gibba* strain G3 callus induction, whereas a consecutive feeding of galactose and sucrose was the best for *L. gibba* var. Hurfeish callus induction (Li et al., 2004). In this study, 2% sucrose was the best carbon source for callus induction in *L. punctata*. Callus induction in other duckweed species such as *S. polyrhiza, S. oligorrhiza, W. arrhiza*, and *L. minor* was also reported. Callus was successfully induced from 91–95% of the explants of *S. oligorrhiza* SP on callus induction media having 2% sorbitol+1% maltose. In the case of *W. arrhiza*, callus induction was supported by callus induction media having 0.7% sorbitol + 0.7% mannitol+2.6% glucose. In the same study, it was shown that 97% of the frond of *W. arrhiza* develop callus when intact plants were grown on Schenk & Hildebrandt (SH) medium supplemented with 2% sucrose+5.0 mg/L 2,4-D+0.5 mg/L 6-BA, after an incubation period of four months. (Khvatkov et al., 2015) The frequency of callus induction in *L. gibba* (MS media containing 2,4-D (20-50 µM)) and *L. minor* (MS media containing 2,4-D (45 µM) were 10 and 89.11% respectively (Moon and Stomp, 1997; Stefaniak et al., 2002).

According to previous studies, the response of explants to different types of growth media, carbon sources and plant growth regulators are species- specific. In case of *L. gibba*, Gamborg's B5 media having 1% sucrose and plant growth regulators (2mg/l picloram or 50mg/L 2,4-D) were the best suited combination for callus growth (Li et al., 2004). The authors also showed that the callus growth of *S. polyrhiza* and *S. punctata* was supported WP medium compared to other basal media used in the experiment. WPM was effective for the entire *in vitro* plant regeneration process in *S. polyrhiza*. In *S. punctata*, WP medium supplemented with 2% sorbitol+ 2,4-D(1mg/L) + NAA (5mg/L) + TDZ (0.5mg/L) supported the callus growth (Li et al. 2004). In the case of *W. arrhiza*, SH media containing 2% sucrose and (4 mg/l picloram) supported the callus growth (Khvatkov et al., 2015). In the present study, the callus growth was promoted significantly on MS medium supplemented with a 2% sorbitol+2,4-D (30µM) +TDZ (1µM). In *S. polyrhiza* callus growth was maximum (98%) on MS media containing 1% sorbitol and 1µM 2,4-D (Wang et al., 2016). It was also shown that 2% sorbitol and 1% maltose was not having any significant effect on callus growth in *S. oligirrhiza* and in the presence of other carbon sources like, glucose, fructose and sucrose mortality rate was 100%. Our study on *L. punctata* shows that 2% sorbitol had a significant role in callus growth, which was similar to the callus growth reported in the same species before (Li et al., 2004).

Plant regeneration from the calli depend mainly on the phytohormones used in the study. MS medium with 1 mg/l TDZ, in *S. oligorrhiza* SP gave 90–100% of the calli regenerated into plants (Li et al., 2004). In the same study, *S. punctata* plant regeneration from the calli was observed on WPM with a combination of carbon source (0.5% sucrose+1% sorbitol) and the cytokinin 2iP. The caron source combination (1% sucrose +2% sorbitol) along with the cytokinin TDZ gave efficient plant regeneration in the present study. In case of *L. gibba* about 55% plant regeneration were seen on Gamborg'sB5 medium with 1mg/l TDZ (Li et al., 2004). In *W. arrhiza*, a PGR free frond

regeneration medium promoted complete plant regeneration (Khvatkov et al., 2015). The tendency of *L. gibba* G3 and *W. arrhiza* fronds to regenerate in a PGR-free condition has previously been reported (Moon and Yang, 2002). When BAP was used in our study as the cytokinin for plant regeneration, 60% regeneration rate was obtained with both the set of experiments but around 3-4 weeks of incubation was needed in plant regeneration medium. The use of MS medium on plant regeneration of *S. punctata* was also reported by Huang et al., 2016. The plant hormone BAP was used in that study along with the carbon source combination (Sucrose (0.5%) + sorbitol (1%)) and obtained a regeneration frequency of 90%. These observations are similar to the results obtained in the present study.

IV. CONCLUSIONS

The search for cost effective methods of recombinant protein expression always kept plants as an alternate expression platform other that mammalian cells or microbial cells. Considering this commercial importance of duckweeds, it is necessary to develop *in vitro* plant regeneration protocols if we want to use them in the industry as a molecular farming platform. Unlike in the case of higher plants like Arabidopsis or Oryza, where one established protocol works with different species across the globe, the plant regeneration protocols of duckweeds, was found to be very difficult to reproduce when we have tried it in our laboratory. So, in the present work we have developed an *in vitro* plant regeneration protocol for the *L. punctata* strain collected from the departmental collection. While the results at some stages resembles the previously published results, this will be the most suitable one for the *in vitro* plant regeneration of this species from this location.

V. EXPERIMENTAL SECTION

A. EXPLANT

Landoltia punctata plants used in this study were collected from the characterized collection in the Botanical Garden, Banaras Hindu University, Varanasi, India (25° 16′ 3.6″ N, 82° 59′ 20.7″ E, and 81 m above sea level), and maintained in the lab under aseptic condition. All the reagents, chemicals, and plant growth regulators are procured from different commercial sources (Merck and Sigma, USA).

B. SURFACE STERILIZATION AND MAINTENANCE OF THE PLANTS

The plants collected from the culture tanks were washed thoroughly with tap water in a conical flask followed by washing with autoclaved sterilized distilled water for five times in the laminar air flow. Surface sterilization of the plants was done with a combination of 0.1 % $HgCl_2$ (for 3, 5 and 5 min) and 70% ethanol (2, 3 and 4 min) followed by washing with sterilized autoclaved distilled water for 6 times of 4 minutes each. The plants were then transferred to a 250 ml flask containing 100 ml half strength Hunter's media (Hutner et al., 1950) supplemented with 1% yeast extract and kept in the tissue culture storage room under ideal culture storage conditions (24±2°C, 14/10 day-night photoperiod, and 30µmol/m^2/s light intensity). The efficiency of surface sterilization under different sterilization conditions were analyzed by the contamination rate in the yeast supplemented medium. The sterilant combination which gave no/very less contamination and maximum plant survival in the presence of yeast extract was selected for further surface sterilization procedures. The decontaminated plants were later transferred to half strength hunter's media in a 500 ml flask and maintained under aseptic condition. Periodic subculture was done once the plants cover the surface of the medium in the flask.

C. CALLUS INDUCTION

For the callus induction, Gamborg's B5 (Gamborg et al., 1968) media with 2% carbon source (sucrose/sorbitol) was used. The media was supplemented with various concentrations and combinations of plant growth regulators, which include Benzyl amino purine (BAP), Thidiazuron (TDZ), 2,4- Dichlorophenoxyacetic acid (2,4-D), and Dicamba (DIC). For making the culture media, about 25 ml of sterilized Gamborg's B5 media was poured into an autoclaved 9-cm glass petri dish and allowed to solidify in the laminar air flow. Mature, mother fronds (2-3 weeks old) from aseptic maintenance media were picked up and the roots and daughter fronds dissected out manually using autoclaved forceps. These fronds were treated as explants for callus induction and transferred to the Gamberg's B5 media in the plates with their abaxial face downwards to the surface of media, and sealed with parafilm. All the cultures of *L. punctata* were maintained at 24±2°C for two-three weeks. After two weeks incubation period explants turned into green calli. The callus induction efficiency of the media was calculated after two weeks. The best responding media were selected and used for further callus induction experiments. All the experiments were replicated three times.

D. CALLUS GROWTH AND MAINTENANCE

Calli induced in the callus induction medium (Gamborg's B5 media + 2% sucrose + 20µM DIC + 1µM BAP) were transferred to the MS (Murashige and Skoog,1962) media supplemented with various phytohormone combinations (2,4-D, DIC, TDZ, BAP) and different concentrations of carbon source (sucrose/sorbitol/mannitol) in the media prepared in a 9-cm glass petri dish. All the Petri dishes having MS media with calli were incubated in the aseptic growth chamber at a 14h/day photoperiod, 24±2°C for three- four - weeks. All the experiments were replicated three times. For the maintenance of the calli, it was subcultured on callus maintenance media with a

combination of TDZ and 2,4 - D (1 and 30μM respectively) under 14h/day photoperiod in a growth chamber at 24±2°C. The calli were transferred to the same media on every two weeks interval.

E. FROND REGENERATION

After four weeks of incubation, calli (5-8 mm in diameter) were transferred to frond regeneration media containing either 3% sucrose or a combination of sucrose and sorbitol (1:2 %) (m/v), and various concentrations of the plant hormones TDZ and BAP. Gamborg's B5 and MS media were used to evaluate the plant regeneration potential of the Calli. The fronds were developed from the calli within two – three weeks. The fully developed individual fronds were separated and cultured on basal MS media developed roots within a week. Once the roots developed the plants were transferred to Erlenmeyer flasks containing 100 mL half-strength Hunters' solution supplemented with 1% sucrose (m/v).

F. STATISTICAL ANALYSIS

All the experiments on callus induction, callus growth, maintenance, and frond regeneration were repeated at least three times. For callus induction studies, 20-25 fronds were placed in each petri dish containing medium supplemented with various phytohormones. For plant regeneration, 15-20 calli were cultured with each of the phytohormone combination used in the experiment. Response percentage was calculated as the total number of calli, which make a new plant divided by the total number of calli transferred in the callus growth media. All the data were analyzed with a one-way analysis of variance (ANOVA) test.

VI. ACKNOWLEDGEMENTS

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VII. DECLARATION

The authors declare no conflict of interest.

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