The Pharma Innovation Journal 2019; 8(9): 255-258

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(9): 255-258

www.thepharmajournal.com
Received: 22-07-2019
Accepted: 24-08-2019

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Changes in biochemical contents of *in-vitro* regenerated callus and plantlets of Japanese Iris (*Iris ensata*)

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Abstract
The growth of *in-vitro* regenerated callus and well rooted plantlets from Japanese Iris leaf tissues was analyzed biochemically. Since enzymes are known as metabolic markers, it changes during development and differentiation. Certain proteins and enzymes are responsible for callus proliferation and differentiation into shoot buds during morphogenesis. In the experiment conducted for two consecutive years, MS basal medium supplemented with 2,4-D (1-2 mg/l) and TDZ (0.1 mg/l) produced the maximum protein, peroxidase and polyphenol oxidase contents in the 30 days old calli. In the well *in-vitro* regenerated plantlets, maximum protein content was observed in the basal medium containing only IBA (3 mg/l), while the combination of both IBA (1-2 mg/l) and NAA (1.0 mg/l) yielded higher peroxidase content and polyphenol oxidase content.

Keywords: Biochemical, callus, plantlets, Japanese Iris (*Iris ensata*)

Introduction
The genus Iris belongs to the plant family Iridaceae, is a winter hardy, herbaceous ornamental, consists of approximately 300 species of flowering plants with showy flowers (Waddick and Zhao, 1992) [24]. Irises are generally propagated vegetatively through splitting of rhizomes or bulbs, but with a very low propagation rate (Jehan *et al*., 1994) [9]. Japanese Iris (*Iris ensata*), native to Japan, China, Korea and Russia, is widely cultivated as an ornamental landscaping and potted plant. It is propagated by division which is a very slow process because they produce small number of plantlets each year (Burnes, 2003) [1]. Propagation through seeds cannot keep genetic uniformity, since the species is an outcrossing one (Yabuya *et al*., 1991) [25]. As propagation through splitting of rhizomes in irises is slow, therefore, micropropagation might be the method of choice (Hussey, 1975; Jehan *et al*., 1994) [6, 9]. Hence, for rapid generation of a large lot of disease-free, true-to-type, uniform, elite, quality planting material - tissue culture technique is applied widely in garden species. The feasibility of *in-vitro* plant regeneration of Iris was first demonstrated by Fujino *et al*. (1972) [7].

Organogenesis relies on the production of organs, either directly from explants or from a callus culture. It relies on the inherent plasticity of plant tissues, and is regulated by altering the components of the medium. Organogenesis in plant tissue culture begins with changes in a single or small group of parenchymatous cells, which then divide to produce a globular mass of cells or meristemoid. These cells can give rise to either a shoot or root primordium under the influence of specific plant bio-regulator (s). Fatima *et al*. (2009) [4] reported that in addition to the influence of plant growth regulators, enzyme activity had also marked effect on the various stages of growth and development during plant regeneration *in-vitro*.

Biochemical attributes are indicators of morphogenetic potential, growth and differentiation, representing differential gene action or expression or change in endogenous level of growth regulators in cell cultures (Carrillo-Castaneda and Mata, 2000) [2]. Marked reductions in the number of biochemical attributes such as starch, protein, amylase, invertase, malate dehydrogenase, peroxidase and phosphorylase with a subsequent increase in soluble sugar and amino acid content lead to *in-vitro* shoot differentiation process (Verghese and Kour, 1991) [23].

Biochemical changes that precede the onset of organogenesis/embryogenesis can serve as markers of differentiation processes that bring about morphological, developmental and functional specialization (Thorpe, 1990). The role of antioxidant enzymes during organogenesis and somatic embryogenesis in some species has been studied in recent years (Mathur *et al.*, 2008, Gupta and Datta 2003, Meratan *et al*., 2009; Misra *et al.*, 2010; Sharifi and Ebrahimzadeh 2010) [11, 5, 13, 14, 20].

During morphogenesis certain enzymes and proteins are responsible for callus proliferation and differentiation into shoot buds (Chawala, 1991).

Materials and Methods

The experiment was conducted for two consecutive years *viz*., March, 2014- April, 2016 at the Plant Tissue Culture Laboratory, Faculty of Horticulture, Uttar Banga Krishi Viswavidyalaya, Pundibari, Cooch Behar, West Bengal, India. The following procedures were followed to record the biochemical content in the fresh calli and the well rooted *in-vitro* grown plantlets:

Total soluble proteins: One gram of *in-vitro* produced callus and plantlet tissues of Japanese Iris were crushed in a chilled mortar with pestle in 5 ml of sodium phosphate buffer (pH 7.5). The ground tissue was centrifuged at 4 ^{0}C for 15 minutes at 10,000 rpm and the supernatant was used as crude protein. It was immediately stored frozen for further use. Total protein was estimated following Lowry's method (1951).

The following chemical were prepared and stored:

Reagent A:	1g Na_2CO_3 in 50 ml of 0.1 (N) NaOH.
Reagent B:	0.25g $CaSO_4$, 5H20 in 50 ml of 1% potassium sodium tartarate.
Reagent C:	50 ml reagent A + 1 ml reagent B.
Reagent D:	1(N) Folin- Ciocaltaeu reagent.

During estimation 0.5 ml protein extract was pipetted into a test tube and 0.5 ml of distilled water was added. The volume was made up to 1 ml. To this, 5 ml of reagent C was added, vortexed and left for 10 minutes. Thereafter, 0.5 ml of reagent D was added, vortexed well and incubated in dark for 30 min. The optical density was measured at 660 nm in a spectrophotometer against a reagent blank without protein extract. Quantity of protein was calculated with the help of the standard curve prepared with Bovine serum albumin (BSA) and expressed as mg/g fresh weight of tissue.

Peroxidase: The extraction of the enzyme peroxidase was carried out by the method of Sadasivam and Manickam (1996) with some modification. Briefly, 0.2 g of fresh plant leaves taken in a precooled mortar and pestle 2 ml of phosphate buffer (pH 7.0, 0.1 M) was added and crushed properly. The homogenate was centrifuged at 10,000 rpm at 4 ^{0}C for 30 minutes in an ultracentrifuge and the supernatant was used as enzyme source. It was stored on ice till the assay was carried out. The activity of peroxidase was assayed by the method of Neog *et al*. (2004). The assay mixture containing 2.5 ml of phosphate buffer (pH 6.5, 0.1 M), 0.2 ml of enzyme extract and 0.1 ml of o-dianisidine (1 mg ml-1 in methanol) was incubated at 28 ^{0}C in a water bath for 2 minutes. The reaction was started by adding 0.2 ml of H_2O_2 (0.2 M). The optical density was recorded at 430 nm at 30 second interval for 5 minutes in a UV-VIS spectrophotometer. The enzyme activity was expressed as "OD min^{-1} g^{-1} fresh weight".

Table 1: Effect of culture media on biochemical content of 30 days old calli of Japanese Iris (*Iris ensata* Thub.)

Treatments	Total soluble Protein content (mg/g)	Peroxidase content (OD min^{-1} g^{-1})	Polyphenol oxidase content (OD min^{-1} g^{-1})
	0.036	0.440	0.172
MS+1.0 mg/l 2,4-D	4.653	5.448	0.907
MS+2.0 mg/l 2,4-D	5.896	4.203	1.077
MS+3.0 mg/l 2,4-D	1.194	2.985	0.558
MS+4.0 mg/l 2,4-D	0.218	1.446	0.464
MS+1.0 mg/l 2,4-D + 0.1 mg/l TDZ	6.327	7.302	1.116
MS+1.0 mg/l 2,4-D + 0.2 mg/l TDZ	3.748	7.080	1.164
MS+2.0 mg/l 2,4-D + 0.1 mg/l TDZ	2.494	8.611	1.307
MS+2.0 mg/l 2,4-D + 0.2 mg/l TDZ	1.769	6.294	1.102
MS+3.0 mg/l 2,4-D + 0.1 mg/l TDZ	2.092	4.476	0.753
MS+3.0 mg/l 2,4-D + 0.2 mg/l TDZ	1.575	3.269	0.647
MS+4.0 mg/l 2,4-D + 0.1 mg/l TDZ	0.955	3.083	0.515
MS+4.0 mg/l 2,4-D + 0.2 mg/l TDZ	0.660	1.796	0.492
S.E(m)±	0.21	0. 22	0.04
C.D. at 5%	0.83	0.87	0.17

Polyphenol oxidase: The assay of the enzyme polyphenol oxidase was done by the method of Mayer *et al*. (1965). Briefly, 0.2 g of finely cut and well mixed fresh leaf was crushed in pre-chilled pestle with the addition of 2 ml phosphate buffer (0.1 M, pH 6.6). The homogenate was centrifuged in an ultracentrifuge at 10,000 rpm for 30 minutes and the supernatant was used as enzyme source. The extract was kept on ice till assay was carried out. The polyphenol oxidase was assayed using pyragallol as substrate. The assay mixture was prepared by taking 100 µl freshly prepared enzyme extract, 2,4 ml of phosphate buffer (pH 6.0, 0.1M) and 0.5 ml pyragallol (0.05M) and the optical density was recorded at 495 nm at 30 second intervals for 5 minutes in a UV-VIS spectrophotometer. The enzyme activity was expressed as "OD min^{-1} g^{-1} fresh weight".

Table 2. Effect of culture media on biochemical content of Japanese Iris (*Iris ensata* Thub.) plantlets developed *in-vitro*

Treatments	Protein content (mg/g)	Peroxidase content (OD min^{-1} g^{-1})	Polyphenol oxidase content (OD min^{-1} g^{-1})
	1.746	0.264	0.360
MS+1.0 mg/l IBA	7.113	3.693	2.812
MS+1.5 mg/l IBA	7.541	2.740	3.098
MS+2.0 mg/l IBA	4.712	2.011	1.246
MS+2.5 mg/l IBA	2.040	0.723	0.888
MS+3.0 mg/l IBA	8.007	6.272	3.970
MS+3.5 mg/l IBA	6.941	5.898	4.208
MS+1.0 mg/l IBA + 1.0 mg/l NAA	6.540	6.841	5.312
MS+1.5 mg/l IBA + 1.0 mg/l NAA	6.248	4.667	3.424
MS+2.0 mg/l IBA + 1.0 mg/l NAA	6.144	3.099	2.636
MS+2.5 mg/l IBA + 1.0 mg/l NAA	5.270	2.414	1.620
MS+3.0 mg/l IBA + 1.0 mg/l NAA	4.143	2.302	1.085
MS+3.5 mg/l IBA + 1.0 mg/l NAA	4.077	1.013	0.975
S.E(m)±	0.23	0.21	0.14
C.D. at 5%	0.91	0.84	0.55

Results and Discussions

The results obtained from Table 1 revealed that MS supplemented with 1.0 mg/l 2,4-D and 0.1 mg/l TDZ produced the maximum content of protein (6.327 mg/g) in the 30 days old cultured calli of Japanese Iris, while MS basal medium fortified with 2.0 mg/l 2,4-D and 0.1 mg/l TDZ produced the maximum peroxidase content (8.611 OD min^{-1} g^{-1}). Highest polyphenol oxidase content (1.307 OD min^{-1} g^{-1}) in the 30 days old fresh calli was recorded in the basal medium containing 2.0 mg/l 2,4-D and 0.1 mg/l TDZ. Basal medium devoid of any plant hormones recorded the least protein content (0.036 mg/g), peroxidase content (0.440 OD min^{-1} g^{-1}) as well as polyphenol oxidase content (0.172 OD min^{-1} g^{-1}).

Data from Table 2 showed that MS basal medium containing 3.0 mg/l IBA recorded the maximum protein content (8.007 mg/g) in the well rooted *in-vitro* regenerated Japanese Iris plantlets, while the maximum peroxidase content (6.841OD min^{-1} g^{-1}) was recorded in the basal medium containing 1.0 mg/l IBA + 1.0 mg/l NAA. Polyphenol oxidase content (5.312 OD min^{-1} g^{-1}) was found highest in the basal medium containing MS + 2.0 mg/l 2,4-D + 0.1 mg/l TDZ. Amongst all the treatments, the least protein content (1.746 mg/g), peroxidase content (0.264 OD min^{-1} g^{-1}) and polyphenol oxidase content (0.360 OD min-1 g-1) was recorded in the hormone free MS medium.

Proteins are important biomolecules having multifarious uses within living organism resulting growth and development. Protein contains amino acids which are precursors of several plant bioregulators namely tryptofan is a precursor of IAA. Higher protein synthesis in calli influenced by a particular culture media may result increased biosynthesis of plant growth promoting substances that results differentiation from callus. The changes in protein content from calli to regenerated plantlets might be due to higher metabolic activities within the regenerated plantlets, thereby synthesis of higher proteins (Niknam and Ebrahimzadeh, 2010) [17]. Some specific reports in this regard have been observed. In *Psophocarpus tetragonolobus* calli, higher protein synthesis was observed with a combination of 2,4-D and TDZ (Naik *et al*., 2015). Meratan *et al*. (2009), Jana and Sekhawat (2012) [13, 8] reported higher concentration of protein in regenerated plantlets of *Acanthophyllum sordidum*, *Anethum graveolens* using 2.69 µM NAA+4.54 µM TDZ+ 2.46 µM IBA and 2.2 µM BA+1.85 µM kinetin respectively in culture media. Enzymes are biocatalyst that accelerate metabolic reactions, ultimately develops metabolites which become useful for plant growth and development. Enhancement of polyphenol oxidase and peroxidase content in calli and regenerated plantlets using plant bioregulators was observed by Tyagi and Swarnkar (1995) [22] in *Arachis hypogea*; Gupta and Dutta (2003) [5] in *Gladiolus hybridus*; Rao and Jabin (2013) [18] in Sugarcane. Lowest protein, polyphenol oxidase and peroxidase content were observed with hormone free MS medium might be due to supply of only essential nutrients but lack of phytohormones to utilize those in the growth and developmental processes towards a definite direction.

Conclusion

Plant tissue culture is one of the most important factors in plant biotechnology for rapid production of elite clones. Evaluation of biochemical behavior produced during the callus growing season as well as the rooted plantlets is essential since they play a major role not only in plant growth, but also on the resistance ability of the regenerated plantlets. From the experiment, it can be concluded that the rate of total soluble protein, peroxidase and polyphenol oxidase production increases with the advancement of plant growth. The biochemical content in both calli and rooted plantlets also depend on the phytohormones used in the basal medium.

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