

## Early Studies on Protoplast Isolation of *Ludisia discolor*, A Wild Orchid

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**Abstract:** The terrestrial *Ludisia discolor*, also referred to as the jewel orchid is prized for the quality of its leaves. *L. discolor* is known as a medicinal herb and is touted for its heat- and pathogen-resisting qualities. *L. discolor* is valuable in the production of both flavonoids and anthocyanins, antioxidants that are exalted in the health industry. Plant cell cultures have emerged as alternative sources of anthocyanin production. Plant protoplast cultures are used frequently in transient gene expression studies and in the establishment of callus and cell suspension cultures. Benefits of plant protoplast system include similarity to cells found in plant tissues, reproduction under controlled conditions, and prevention of masking of stress responses to previous handling techniques. A study was conducted to assess the amenability of the stem and leaves of *L. discolor* to protoplast isolation. The stem and leaf segments were weighed, sliced into thin layers, immersed in a digestion medium, washed and then cultured onto a recovery medium. Results indicated that the production of plant protoplasts from *L. discolor* may be viewed as an alternative in the generation of cell cultures and ultimately in the production of anthocyanins from the cell cultures.

**Keywords:** Anthocyanins, Cell Culture, Jewel Orchids, *Ludisia discolor*, Protoplast Isolation

## INTRODUCTION

The terrestrial and medicinal *Ludisia discolor*, a jewel orchid, is prized for the quality of its leaves. *L. discolor* is used for its heat- and pathogen-resisting qualities, attributed partly to its amino acids and anthocyanin content (Shiau *et al.* 2002). Studies have indicated that anthocyanins help protect chloroplasts against high light intensities and prevent photoinhibition by masking the chlorophyll-containing plant organelles (Pietrini *et al.* 2002; Stintzing & Carle 2004).

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Protoplasts are plant cells in which the cell wall has been degraded enzymatically or mechanically. Protoplasts are theoretically totipotent, having the ability to dedifferentiate, re-enter cell cycles, undergo repeated mitotic divisions, and proliferate or regenerate into various organs (Johnson & Veilleux 2001; Eckhaut *et al.* 2013). Protoplasts are good models to study physiological processes in plant cells (Horváth 2009).

The objectives of this study were to assess the amenability of stem and leaves of *L. discolor* to protoplast isolation, and to enumerate the effects of various isolation parameters on protoplast harvest rates.

## MATERIALS AND METHODS

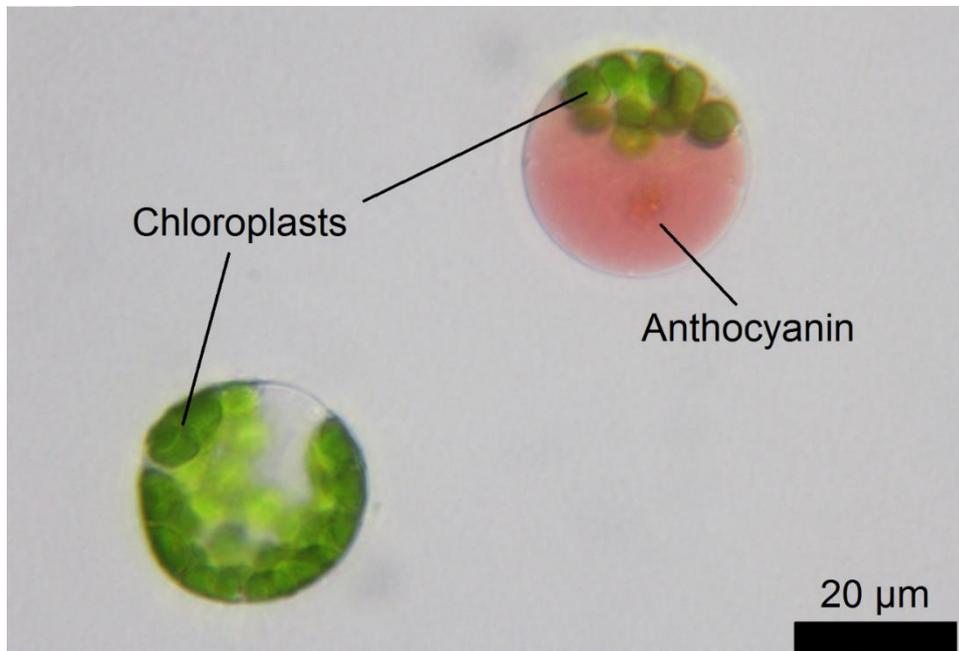
The leaves and stem of 6 month old *in vitro* plantlets of *L. discolor* (Fig. 1) were used in this study. One gram of excised stem and leaves were either directly sliced into 0.5–1.0 mm parts and placed in digestive medium composed of 2% (w/v) cellulose and 0.5% (w/v) macerozyme (Yoo *et al.* 2007); or placed in an osmotic medium prior to slicing and placement in the digestive medium (Nagy & Maliga 1976; Horvát 2009). The plant tissue segments were digested for between 15 to 20 hours. The protoplast mixture was then centrifuged at either 750 or 1000 rpm, and the pellet suspended twice in a washing solution. After the final rinse, the pellet was suspended in a buffer solution, and incubated at 4°C for 30 minutes. Protoplast number and viability were enumerated in this period using Evans Blue solution. Means were analysed through one-way ANOVA and differentiated with Tukey's test, with the probability value set at 0.05.



**Figure 1:** An *in vitro* *L. discolor* plant.  
Note: Bar = 1.0 cm.

## RESULTS AND DISCUSSION

Protoplast isolation was observed to be successful when performed on *in vitro* segments as opposed to *ex vitro* segments, and on leaves compared to stems. Many of the leaf protoplasts contained anthocyanins, detected as a rich shade of red in the cytoplasm of the cells (Fig. 2). In the case of *Brassica oleracea*, protoplasts could be isolated from the mesophyll tissues of all accessions tested. Anthocyanins in the bright green and purple colour ranges were detected in protoplasts isolated from red-coloured *B. oleracea* cultivars (Kielkowska & Adamus 2012). Protoplasts from white forms and Savoy cabbage were spherical and rich in chloroplasts that were randomly distributed in the cytosol (Kielkowska & Adamus 2012), as observed in this study (Fig. 2). However, isolated stem protoplasts of *L. discolor* were observed to be lysed during cell counting, possibly due to the penetration of raphides during the centrifugation steps. Stem protoplast harvest improved with increased stem fresh weight.



**Figure 2:** Viable protoplasts isolated from *in vitro* leaves of *L. discolor*.  
Note: Bar = 20  $\mu\text{m}$ .

The protoplast yield in this study varied between the explants used, and the parameters assessed, with higher yields obtained from leaf explants. Initial experiments indicated that the centrifugation speeds selected did not affect protoplast yield significantly; hence 750 rpm was used in subsequent experiments (data not shown). Protoplast yield improved with a pre-isolation

osmotic treatment and the use of K3 medium, when compared to direct digestion in a digestive medium (data not shown). No significant difference was detected among the digestion periods tested. A harvest of up to  $7.7 \times 10^6$  cells could be obtained when the leaves were digested for 15 hours (Table 1), with viability percentages in all treatments ranging from 46%–57%, as assessed through the Evans Blue assay. This number is comparable to that obtained from *B. oleracea*, in which the average yield of protoplasts obtained from all accessions was  $2.0 \pm 0.1 \times 10^6$  per gram of fresh tissue. The protoplast yield was attributed to the cultivar tested and the age of the leaf tissue sources.

**Table 1:** Effect of various digestion hours on the viability and number of protoplasts harvested from *in vitro* leaves of *L. discolor*.

Digestion period (hours)	Total cells harvested	Total viable cell harvested	Percentage of viable cells
15	$7.7 \pm 4.0 \times 10^6$	$3.7 \pm 2.0 \times 10^6$	46.6 ± 7.4
16	$5.0 \pm 0.0 \times 10^6$	$2.9 \pm 0.0 \times 10^6$	57.0 ± 0.0
18	$2.9 \pm 0.7 \times 10^6$	$1.5 \pm 0.6 \times 10^6$	51.6 ± 8.4
20	$5.6 \pm 0.4 \times 10^6$	$2.8 \pm 0.6 \times 10^6$	49.2 ± 7.4

## CONCLUSION

The study indicates that *in vitro* plantlets of *L. discolor* are amenable to protoplast isolation. This presents a viable option in the generation of cell cultures targeting the production of anthocyanins. More parameters need to be assessed in order to improve protoplast isolation and regeneration rates for this wild orchid.

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