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Multiple Shoot Regeneration of an Important Equatorial Forage Plant *Indigofera zollingeriana* Miq.

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ABSTRACT

Indigofera zollingeriana is an important protein rich forage and dye plant species that grows widely in Indonesia. It is difficult to propagate the plant through seeds due to high dormancy. Therefore, there is need to develop both *in vitro* and *ex vitro* propagation techniques of *I. zollingeriana*. This study aimed to develop a protocol for multiple shoot regeneration of the plant under *in vitro* conditions using different combinations of BAP+0.01 mg/L NAA. The results showed that both epigeal and cotyledon node explants have high potential to regenerate with maximum number of regenerated shoots noted on the epigeal and the cotyledon node explants using MS medium containing 1.5 mg/L BAP + 0.01 mg/L of NAA and 1.0 mg/L BAP + 0.01 mg/L of NAA in the same order. The shoots regenerated on all combinations of BAP+NAA were rooted using 0.5 mg/L IBA that promoted rooting in effective manner. The rooted plants were easily acclimatized to external conditions in an environmental chamber. The results suggest that *I. zollengriana* plant species is no more difficult to regenerate under *in vitro* conditions. This research will aid in future studies related to breeding, propagation, biosynthesis and extraction of active compounds.

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Önemli Bir Ekvator Yem Bitkisi Olan *Indigofera zollingerian* Miq.'nın Çoklu Sürgün Rejenerasyonu

ÖZET

Indigofera zollingeriana, Endonezya'da yaygın olarak yetişen proteince zengin yem ve boya bitkisidir. Bitkiyi tohumdan çoğaltmak yüksek dormansi sebebiyle zor olmaktadır. Bu nedenle, *I. zollingeriana*'nın *in vitro* ve *ex vitro* koşulllarda çoğaltma tekniklerinin geliştirilmesi gerekmektedir. Bu çalışmada, farklı kombinasyonlarda BAP + 0.01 mg/L NAA içeren MS ortamı kullanılarak *in vitro* koşullar altında bitkinin hızlı çoğaltım için bir protokol geliştirmesi amaçlanmıştır. En fazla sürgün rejenerasyonu epigeal ve kotiledon nodlarının sırasıyla 1.5 mg / L BAP + 0.01 mg / L NAA ve 1.0 mg/L BAP + 0.01 mg/L NAA içeren MS ortamında elde edilmiştir. BAP + NAA'nın tüm kombinasyonlarında rejenerasyonu yapılan sürgünler 0.5 mg/L IBA kullanılarak başarılı şekilde köklendirilmiştir. Köklü bitkiler, bir iklim dolabında dış koşullara alıştırılmıştır. Bu çalışma *I. zollingeriana* bitkisinin *in vitro* koşullar altında yetiştirilebileceğini göstermiş olması ve elde edilen sonuçların ilerideki çalışmalarda aktif bileşiklerin biyosentezi ve ekstraksiyonu ile bitki yetiştirilmesi ve çoğaltılmasına yardımcı olacağını düşünülmektedir.

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INTRODUCTION

Indonesia makes 1.3% of the world's land surface, consisting of more than 18000 islands with third largest area under rainforests and is home to 10

percent of the world's known plant species. This is very high biological diversity after the Amazon and the Congo Basin. *Indigofera zollingeriana* with rich protein is an important forage plant species (Abdullah, 2010) that grows throughout Indonesia especially in Bali, Bangka, Flores, Jawa, Kangean Maluku and Sulawesi area (Wiriadinata, 2012).

The leaves of plant are used to extract indigo dye and manufacture of variable pharmaceutics and cosmetics. Its leaves have high dry matter and protein (27 to 31%) of which 75 to 87% is digestible, with neutral detergent fiber of 49-57% and acid detergent fiber of 32-38%, with low total tannin contents (Abdullah, 2010). When used as forage, it improves meat and milk quality of dairy goat and Indonesian local sheep (Apdini, 2011; Abdullah et al., 2012; Pardede, 2012; Sambas, 2012; Retnani et al., 2013; Baihaqi et al., 2014; Retnani et al., 2014). *I. zollingeriana* top leaf meal has also been reported to act as substitute of soybean protein meal in poultry feed that improve quality of chicken meat, layer hens and quail eggs (Palupi et al., 2014; Faradillah et al., 2015; Santi et al., 2015). Marina (2012) and Nofesa (2012) demonstrates that *I. zollingeriana* leaf pellet forms affect sperm quality of New Zealand White crossbred rabbit positively.

It is known that the plant has low rate of propagation from the seeds. Each *I. zollingeriana* seedpod has 5-7 small seeds with high dormancy that increases with the passage of time such that the seeds stored for 2 months has viability of only 28-35%. (Abdullah et al, 2012; Abdullah 2014).The plant is highly tolerant to drought and has forage production in range of 7-10 ton/ha/harvest with foliar fertilizer application (Abdullah, 2010).

A few *in vitro* culture studies have been reported for other *Indigofera* species like *I. enneaphylla*, *I. potaninii*, *I. tinctoria, I. viscosa* and *I. tirta* (Bharal and Rashid, 1978; Howell et al., 1986; Nair and Reghunath, 2009; Rajabudeen et al., 2014; Senthilkumar et al., 2015; Nair et al., 2016). All of them indicated that Indigofera species are highly recalcitrant. Therefore, there is need to develop a micropropagation protocol for *I. zollingeriana*. Objective of this study was to determine the effect of different combinations of plant growth regulators on multiple shoot regeneration of *I. zollingeriana.*

MATERIALS and METHODS

The *I. zollingeriana* seeds used in this study were provided by Prof. Dr. Luki Abdullah (Bogor Agricultural University, Department of Nutrition Science and Feed Technology, Indonesia).

Sterilization and scarification

The seeds were scarified using sulphuric acid treatment for 5 minutes. Subsequently, they were rinsed 3 × 5 minutes with autoclaved bidistilled water. The seeds were shaked at the rate of 190 rpm on thermoshake in distilled water for germination. These geminated seeds were transferred to 0.7% agar solidified and 3% sucrose supplemented MS medium (Murashige and Skoog, 1962), pH 5.6-5.8. Subsequently, the cotyledon and the epigeal node explants were taken from the growing seedlings after two weeks and cultured on MS medium (control treatment) and agar solidified sterile MS medium containing 0.5, 1.0, 1.5, 2.0, 2.5 mg/L BAP + 0.01 mg/L NAA (5 treatments), pH 5.6-5.8 for micro-cloning.

At the end of nine weeks, the regenerated shoots were rooted on MS medium fortified with 0.5 mg/L IBA for 30 days.

The rooted plantlets were transferred to 600 mL plastic pots filled with 500 mL locally prepared leaf peat moss for their acclimatization.

Culture conditions

All *in vitro* and *ex vitro* studies were conducted very carefully under aseptic conditions in sterile growth cabinet maintaining 24±1°C temperature under 16 h light (35 µmol photons m^{-2} s^{-1}) photoperiod.

Statistical Analysis

Each experimental treatment contained 60 explants that were equally distributed into 15 replicates, with 4 explants each. The experimental data was subjected to one-way analysis of variance using computer statistical software "IBM SPSS 24". The comparison among means was made using DMRT. The percentage data was always subjected to arcsine transformation before analyses (Snedecor and Cochran, 1976).

RESULTS

The epigeal and the cotyledon nodes (Fig.1a) isolated from 4 weeks old seedlings showed profuse but variable percentage of compact. Friable callus induction and shoot regeneration (Table 1) were in range of 67 -100% and 58-100%, respectively. Frequency of callus induction on the epigeal node explants on all treatments were statistically non-significant. The maximum callus induction from the cotyledon node was observed on MS medium containing 1.5 and 2.5 mg/L of BAP with 0.01 mg/L NAA (Table 1).

Treatment		Callus induction Percentage (%)		Number of Shoots Per Explant		Shoot Length (cm)		Lateral Shoot Length (cm)		Rooting Percentage (%)	
BAP (mg/L)	NAA (mg/L)	Epigeal Node	Cotyledon Node	Epigeal Node	Cotyledon Node	Epigeal Node	Cotyledon Node	Epigeal Node	Cotyledon Node	Epigeal Node	Cotyledon Node
0.5	0.01	83.00	58.00b	1.50ab	1.58a	1.68ab	3.47a	0.25b	0.48	50.00	50.00
1.0	0.01	75.00	92.00a	1.67ab	2.08a	2.26a	1.78b	1.12a	0.24	33.33	41.67
1.5	0.01	100.00	100.00a	2.17a	1.92a	1.00bc	1.15bc	0.18b	0.07	33.33	33.33
2.0	0.01	75.00	75.00ab	1.17b	1.75a	1.16bc	1.40bc	0.14b	0.11	33.33	16.17
2.5	0.01	92.00	100.00a	1.75ab	1.67a	1.26bc	0.83c	0.18b	0.09	8.33	8.33
Control		67.00	58.00b	1.33b	0.83b	0.64c	0.59c	0.14b	0.25	0.00	25.00

[1]Means not followed by same letter within a column differ significantly at P<0.05

Figure 1 Shoot regeneration of *I. zollingeriana* (a) epigeal and cotyledon node on seedlings. Shoot regeneration from (b) the epigeal and (c) the cotyledon nodes (d, e) Diametrically opposed views of rooting of shoots regenerated on MS medium having 1.5 mg/L of BAP and 0.01 mg/L of NAA using 0.5 mg/L IBA in magenta vessels (f) acclimatized plants

All calli induced browning, if the explants were exposed to BAP containing medium for more than 9 weeks. However, the rate of induction of browning followed by necrosis increased with each increasing concentrations of BAP.

The maximum number of shoots on the epigeal node was obtained on MS medium containing 1.5 mg/L BAP + 0.01 mg/L of NAA, while maximum number of shoots on the cotyledon node regenerated shoots were noted on MS medium having 1.0 mg/L of BAP + 0.01 mg/L of NAA (Fig. 1 b, c). The developing shoots had induction rate of 1.17 to 2.17 and 0.83 -2.08 shoots per explant along with shoot length of 0.64-2.26 cm and 0.59 to 3.47 cm on the epigeal node and the cotyledon node explant, respectively. These shoots induced variable number of lateral shoots. The maximum lateral shoot length of 1.12 cm on the epigeal node. The lateral shoot length on the cotyledon node was not significant and conspicous that ranged from 0.07 to 0.48 cm in length. The shoots induced on all culture treatments on both explants were subjected to rooting using 0.5 mg/L IBA. Irrespective of the explant, maximum roots were noted on the shoots induced on medium containing 0.5 mg/L of BAP + 0.01 mg/L NAA (Table 1). The rate of root induction on shoots induced on the epigeal node explant decreased thereafter, but remained stable on all concentrations of BAP+NAA. However, in case of shoots taken from the cotyledon node explant, the rate of root induction decreased with each increased BAP concentration in the treatment.

Variable rooting was noted on all shoots cultured in their own regeneration treatments. The best rooting (50%) was noted on the regenerated shoots obtained from MS medium containing 0.5 mg/L BAP + 0.01 mg/L NAA using the epigeal and the cotyledon node explants (Table 1). Non rooting shoots noted on MS medium containing 1.0 mg/L of BAP and 0.01 mg/L of NAA (the best shoot regeneration treatment) were rooted using 0.5 mg/L IBA. This concentration of IBA was very effective to promote roots (~75% - not given in the Table) on shoots (the epigeal and the cotyledon node explant) (Fig. 1d, e). The Epigeal node explant induced shoots were better in performance compared to the cotyledon node induced shoots.

All of the plantlets contained in plastic pots (Fig. 1f) were acclimatized in the environmental chamber with 100% survival.

DISCUSSION

In general, legumes are considered as recalcitrant to *in vitro* culture (Tejavathi et al., 2010). The previous studies showed significant progress on the *in vitro* culture of legume and forage legume that are being amenable to *in vitro* plant regeneration than grain legume (Flick et al., 1983; Phillips and Collins, 1984).

Many previous studies report successful regeneration. *In vitro* studies on *Macrotyloma uniflorum*, known horse gram, lentils, cowpea, chickpea, faba bean etc. have already been reported using cotyledon nodes (Aasim et al., 2009; Varisai et al., 1998; Varisai et al., 1999).

Other studies on Indigofera, by Rajabudeen et al. (2014) notified some of shoots of *I. viscosa* were rooted on the medium supplemented with 2 mg/L BAP and 2.5 m/L NAA.

Howell et al. (1986), Ozel et al. (2008, 2015) reported that *I. potaninii*, *Ornithogalum ulophyllum* and *Muscari muscarimi* easily regenerate shoots and bulbs on medium containing high level of both BAP and NAA. The above-mentioned rooting results are fully or partially in agreement with previous studies. The results show no hindrance in rooting of BAP-NAA induced shoots on *I. potaninii.* Nair and Reghunath (2009) and Nair et al. (2016) studied on *I. tinctoria* and noted that *in vitro* axillary shoot proliferation was achieved from single-node explants on MS medium containing various concentrations of 1 mg/L BA and IAA acid. Rajabudeen et al. (2014) reported axillary buds of *I. viscosa* from nodal explants proliferated on 1 mg/L BAP and 1.5 mg/L NAA underwent multiplication of shoots. Furthermore, they noted that both phytohormones were the most effective treatments for promoting shoot multiplication after four weeks on MS medium containing concentrations of BAP + NAA and indicated that 1 mg/L BAP and 1.5 mg/L NAA improved shoot length of regenerated shoots of *I. viscosa* using nodal and shoot tip explants. Senthilkumar et al. (2015) studied on *I. tirta* and the best result on shoot regeneration was using 2 mg/L BAP on MS medium with shoot length (3.9 cm), percentage of shoot proliferation was 96.2%. Other studies by Nair et al. (2016) reported the best shoot proliferation on *I. tinctoria* using nodal explants in MS medium containing 1.0 mg L/1 BA +0.1 mg/L IAA.

Howell et al. (1986) determined the percentage of rooted shoots of *I. potaninii* on B5 medium in the dark at 27°C that increased by culturing shoots in 1.0 mg/L IBA for two to three days before transfer to growth regulator free medium. Rajabudeen et al. (2014) reported 2 mg/L IBA was noted as the best rooting hormone with 81.2% of rooting frequency compared to IAA and NAA for *in vitro* rooting of *I. viscosa* and increasing IBA concentration was not related to increasing of rooting percentage (%). Contrary, Nair et al. (2016) reported that *I. tinctoria* showed the most efficient rooting on MS medium supplemented with 1.5 mg/L IAA compared to IAA and IBA. Nevertheless, adding 0.5 mg/L IBA to MS or ½ MS medium induced slow growth in *I. tinctoria* induced rooting on all cultures compared to ½ MS medium induced rooting and MS medium induced rooting 16.67 %. Senthilkumar et al. (2015) reported that the best root

induction on shoots developed of *I. tirta* were on half strength MS medium with 2 mg/L IBA.

The results of this study demonstrate that it is possible to micropropagate on both explants that were juvenile using rapid, simple and reproducible methodology. However, there is need to improve the protocol described in this study. It should be known that there was no problem in rooting of micropropagate shoots and they were easy to establish in pots after acclimatization. Any combination of BAP+ NAA was variably suitable for regeneration and rooting. However, the epigeal explant was the best in terms of regeneration IBA was root promotive on most o the shoots irrespective of the explant and the phytohormones on which they were regenerated. Generally, 100% survival was noted on all of the rooted plants. This is the first study o tissue culture of *I. zollingeriana* and will be helpful in propagation, breeding studies of the plant and provide a lead to the biosynthesis and extraction of active compounds.

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