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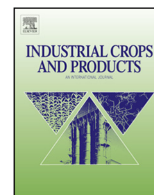

A reproducible and high frequency plant regeneration from mature axillary node explants of *Gymnema sylvestre* (Gurmur)—An important antidiabetic endangered medicinal plant

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ABSTRACT

Gymnema sylvestre is an important medicinal plant used in different systems of medicine as a remedy for the treatment of diabetes. The present study describes an efficient and rapid protocol for large scale *in vitro* plant regeneration from mature axillary node explants of *G. sylvestre*. The axillary node explants were cultured on MS medium supplemented with different concentrations of BAP and KIN (0.5–3.0 mg/l) for shoot bud induction. In order to enhance the shoot bud multiplication, regenerated shoot buds were further subcultured onto MS medium fortified with different concentrations of BAP (0.5–3.0 mg/l) in combination with 0.5 mg/l of NAA/IBA/IAA/KIN. The highest frequency (84.22%) of multiple shoot bud regeneration with maximum number of shoots (14.20 shoots/explant) was noticed on MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l KIN combination. In another experiment, *in vitro* derived shoot buds were cultured on different concentrations of GA_3 (0.5–2.0 mg/l) and various concentrations of BAP (0.5–3.0 mg/l) in combination with KIN (0.5 mg/l) and GA_3 (1.0 mg/l) for shoot bud multiplications as well as elongation. For large scale plant production, *in vitro* derived axillary buds were cultured on MS medium fortified with BAP (1.0 mg/l) + KIN (0.5 mg/l) + GA_3 (1.0 mg/l) combination, in which about 418.72 shoots/explant were obtained after five subcultures on the same media composition. Elongated shoots (>2 cm) were dissected out from the *in vitro* proliferated shoot clumps and were cultured on half-strength MS medium containing different concentrations of various auxins (IAA, IBA and NAA) (0.5–2.0 mg/l) for root induction. Highest frequency of rooting (78%) was noticed on half-strength MS medium augmented with 2.0 mg/l IBA. The rooted plantlets were successfully transferred into plastic cups containing sand and soil in the ratio of 1:2 and subsequently they were established in the greenhouse. The present *in vitro* regeneration protocol would facilitate an alternative method for fast and large scale propagation of this endangered antidiabetic medicinal plant.

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1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia resulting from defective insulin secretion, resistance to insulin action or both (Khan et al., 2012). Diabetes is a deadly disease that affected an estimated 285 million people worldwide in 2010 and the number is increasing in rural and poor populations throughout the world and is projected to become one of the world's main disablers and killers within the next 25 years (Shaw et al., 2010). Hyperglycemia and hyperlipidemia are two important characters of diabetes mellitus, an endocrine based disease. Currently available therapy for diabetes includes insulin and various oral hypoglycemic agents such as sulfonylureas, metformin, glucosidase inhibitors, troglitazone, etc. (Kameswararao et al., 2003). These drugs are used as mono-therapy or in combination to achieve better glycemic control but they have their limitations and are known to produce serious side effects; therefore, the search for safer, specific and effective hypoglycemic agents has continued to be an important area of investigation with natural extracts from readily available traditional medicinal plants offering potentials for discovery of new antidiabetic drugs (Klein et al., 2007). Over the years, various medicinal plants and their extracts have been reported to be effective in the treatment of diabetes. Plants are rich sources of antidiabetic, antihyperlipedemic and antioxidant agents such as flavonoids, gallotannins, amino acids and other related polyphenols (Muruganandan et al., 2005; Miyake et al., 2006).

The major concern is that most of the medicinal plants are collected from the wild populations, and over 70% of the plant collection involves destructive harvesting mainly because of the use of plant parts like leaf, bark, wood and whole plants. Medicinal plants are of great concern to the researches in the field of plant

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biotechnology not only for rapid propagation but also for production of valuable secondary metabolites. Overexploitation of the plant for its various potential applications in the indigenous system of medicine has caused a serious threat to the existence of medicinal plant species. The harvest of medicinal plants on a mass scale from the natural habitat is leading to a depletion of plant resources.

Gymnema sylvestre R. Br. (Asclepiadaceae), a vulnerable species is a slow growing, perennial woody climber of tropical and subtropical regions and it is popularly called as "Gur-mar" for its distinctive property of temporarily destroying the taste of sweetness and is used in the treatment of diabetes. The leaves of the plant in particular are used as antiviral, diuretic, antiallergic, hypoglycemic, hypolipidemic, antibiotic, in stomach pains and in rheumatism. In addition, it possesses antimicrobial, anti-hypercholesterolemic, hepatoprotective and sweet suppressing activities. The various reports on its multiple uses attracted attention for utilization of the plant for gymnemic acid. *G. sylvestre* species is threatened with extinction due to its indiscriminate collection for commercial purposes and to meet the requirements of the pharmaceutical industry. Conventional propagation is hampered due to its poor seed viability, low rate of germination and poor rooting ability of vegetative cuttings. Inevitably, therefore, rapid multiplication of this important drug yielding genotype is imperative. Alternatively, *in vitro* micropropagation would be beneficial in accelerating large scale multiplication and conservation of this important plant species.

In vitro culture methods through multiple shoot induction using axillary node explants have proved successful for rapid propagation of a number of medicinally important species such as *Santolina canescens* (Casado et al., 2002); *Bupleurum fruticosum* (Fraternale et al., 2002); *Rauvolfia tetraphylla* (Faisal et al., 2006). *In vitro* culture allows maintaining clonal fidelity, and also assures the consistent production of true-to-type plants within a short span of time. There are few reports on *in vitro* propagation of *G. sylvestre* using axillary node explants. *In vitro* plant regeneration from nodal explants of *G. sylvestre* was reported by Komalavalli and Rao (2000), Sairam Reddy et al. (1998) and Subathra Devi and Mohana Srinivasan (2008). Though, the plant regeneration was reported in *G. sylvestre* the percent of shoot bud regeneration was found to be low and may not be useful for production of large scale plants for commercial purpose/cultivation. Therefore, development of a rapid protocol for high frequency *in vitro* plant regeneration in this important medicinal herb became necessary in order to reduce the existing pressure on natural populations and continuous supply of plant materials for the pharmaceutical industry. As tissue culture technique has now become a well established method for large scale plants developed for commercial utilization of several endangered medicinal plants. The major goal of the present investigation was to standardize the best media composition, growth regulators combinations for high frequency plantlet production from mature axillary node explants of *Gymenma sylvestre* species. In order to enhance the shoot bud multiplication rate, various growth regulators were examined to identify the best growth hormone combinations for maximum number of shoot buds production. In addition, *in vitro* culture passages were also standardized to maximize the number of shoot buds without losing its vigour and viability for commercial scale application/utilization.

2. Materials and methods

2.1. Preparation of explants

G. sylvestre plants were collected from ABS medicinal garden, Salem, Tamil Nadu and maintained in the Greenhouse, Department of Biotechnology, Periyar University, Salem 11. For shoot bud induction, mature axillary node explants were collected from 3 months old plants and were washed in running tap water. Explants were washed with few drops of 10% (v/v) Tween-20 to remove the superficial dust particles including microbes. Then, they were surface sterilized with 0.1% (w/v) mercuric chloride solution for 10 min followed by rinsing them for five times with sterile distilled water. Sterilized nodal explants were used for *in vitro* culture studies as described below.

2.2. Culture media and growth conditions

The culture medium consisted of MS (Murashige and Skoog's, 1962) salts, vitamins, 3% (w/v) sucrose and the pH of the media was adjusted to 5.6 with 0.1 N NaOH or HCl before adding 0.7% (w/v) agar. Media (15 ml) were poured into 25 mm × 150 mm culture tubes (Borosil, Mumbai) and autoclaved at 121 °C for 15 min. The cultures were maintained at 24 ± 2 °C under 16/8 h (light/dark cycle) photoperiod (60 μE m^{-2} s^{-1}) and irradiance provided by cool-white fluorescent tubes (Philips, India).

2.3. Shoot bud initiation

Surface sterilized mature axillary node explants were cultured on MS medium supplemented with different concentrations of BAP and KIN (0.5–3.0 mg/l) for shoot bud induction. After two weeks of culture, direct shoot bud initiation from the axillary node explants was noticed.

2.4. Induction of multiple shoots

In this experiment, axillary node explants derived *in vitro* regenerated shoot buds as explants source were cultured on MS medium fortified with different concentrations of BAP (0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) in combination with 0.5 mg/l IAA/IBA/NAA/KIN individually for multiple shoot bud development.

2.5. Shoot bud elongation and multiplication

To achieve shoot bud multiplication and elongation, two experiments were adopted. In the first experiment, the *in vitro* derived shoot buds were cultured on MS medium supplemented with different concentrations of GA_3 (0.5–2.0 mg/l) alone for shoot bud elongation. In another experiment the shoot buds were cultured on different concentrations of BAP (0.5–3.0 mg/l) in combination with 0.5 mg/l KIN and 1.0 mg/l GA_3 for shoot bud multiplication and elongation.

2.6. Large scale production of shoot buds

For large scale plant production, *in vitro* regenerated shoot buds were subcultured onto MS medium supplemented with 1.0 mg/l BAP + 0.5 mg/l KIN + 1.0 mg/l GA_3 combination. The cultures were subcultured onto the fresh same media composition once in 2 weeks interval. This process was repeated for another five subcultures (each 15 days) to examine the effect of subculture on production of large scale shoot buds. After 75 days of culture multiple shoots were counted for analysis of total number of regenerated shoot buds.

2.7. Rooting of elongated shoots and acclimatization

The elongated shoots (>2.0 cm height) were transferred to half-strength MS medium fortified with different concentrations of IAA or IBA or NAA (0.5–2.0 mg/l) for root induction. Plantlets with well-developed roots were removed from the culture tubes and gently washed under running tap water to remove adhering medium. Subsequently, they were transferred to plastic cups containing sterile

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