Micropropagation and antioxidant activity of *Mollugo nudicaulis* Lam.

K. S. Nagesh* and C. Shanthamma

Department of Botany, Medicinal Plant Tissue Culture Lab, University of Mysore, Manasagangothri, Mysore – 570 006, Karnataka, India.

Accepted 16 February, 2011

*Mollugo nudicaulis* Lam, is a traditionally important wild medicinal herb, the leaves have been used by traditional practioners for curing whooping cough and jaundice. This plant in nature propagated itself through seeds during rainy season, collection of plant material for extraction of drug is a major task. To overcome these constraints *in vitro* propagation protocol for *M. nudicaulis* was developed using shoot tip culture. Shoot tips were cultured on different types of nutrient medium fortified with different concentrations and combinations of growth regulators. Among these Murashige and Skoog’s (MS) media containing 2 mg/l of BAP alone found to be suitable for multiple shoot induction. Roots were induced on half-strength MS medium containing IBA (1 mg/l). Further antioxidant activity was carried out using natural leaves extracts (NLE) and *in vitro* leaf extracts (ILE) using reducing power and DPPH radical scavenging assays. *In vitro* derived leaf extracts showing more antioxidant activity than NLE. In addition we examined the content of total phenolics in NLE and ILE by FC method. An approximate 1.75 fold increased total phenolic content was observed in *in vitro* derived leaves (2.502 mg/g) than that of natural leaves extract (1.43 mg/g). This increased phenolic content in *in vitro* leaf extracts indicated a good correlation between the levels of phenol and antioxidant activity. Results, suggested that the developed protocol will have a direct impact on the exploitation of biotechnological sources for extraction of large scale antioxidants for medicinal purposes.

Key words: *Mollugo nudicaulis*, multiple shoot induction, shoot tip, antioxidant activity, natural leaves extracts, *in vitro* propagated leaves extracts.

INTRODUCTION

Antioxidants are component, which inhibits oxidation, or free radicals induced oxidative damage and therefore are potential quenchers of oxidative stress induced lipid peroxidation. Antioxidants effectively prevent free radical induced cellular and tissue damage which are solely responsible for several pathogenic condition including cancer (Bandyopadhyya et al., 1999; Gulcin, 2009; Talaz et al., 2009).

Antioxidants are naturally present in biological systems such as plant and plant derived products. Such naturally occurring antioxidants provide a protection against oxidation. Antioxidants are the compounds that when added to food products, especially to lipids and lipid-containing foods, can increase the shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food products during processing and storage. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have restricted use in foods as these synthetic antioxidants are suspected to be carcinogenic (Madhavi and Salunkhe, 1995). Therefore, the importance of the search and exploitation of natural antioxidants, especially of plant origin, has greatly increased in recent years (Gulcin, 2010; Sarikurkcu, 2011).
Anthocyanins, which are widely present in various plant species, having strong antioxidant activity and prevent oxidative damage (Hollaman, 2001). The genus Mollugo (Molluginaceae) are of great medicinal importance, due to presence of anthocyanins (Chopin et al., 1986) and also well known for its antimicrobial activity. Among these, *Mollugo nudicaulis* Lam, is a one such traditionally important wild medicinal herb where the leaves are used by traditional practitioners for curing whooping cough and jaundice (Kirtikar and Basu, 1935) and is reported to contain six different types of saponins (Anonymous, 1962). This plant in nature itself propagated through seeds during rainy season. Furthermore collection of plant material for extraction of drug is a major task, as this small herb grows in localized area and during rainy season. Therefore, there is an urgent need to search for alternative techniques for production of plant material for extraction of drug throughout season. Plant tissue culture techniques play an important role production of plant source as alternative choice for the extraction of drugs (Dias et al., 2000). To the best of our knowledge there are no reports on *in vitro* propagation or antioxidant activity on this species. Therefore the main objectives of this study is to *in vitro* propagation and evaluation antioxidant activity of *M. nudicaulis* natural leaves extracts (NLE) and *in vitro* leaf extracts (ILE) using various *in vitro* models.

**MATERIALS AND METHODS**

**Plant materials**

The *M. nudicaulis* plants were collected from open field in and around Mysore city. The plants were thoroughly washed in running water for 15 min followed by 10% (v/v) liquid detergent, Teepol for 10 min and the excess of detergents was removed by washing the explants thoroughly under running tap water. The explants were surface sterilized using Cetrimide (0.25%) and Ampicillin (0.15%) for 8 to 10 min and disinfected with 1% Mercuric chloride. The explants were washed with sterilized distilled water after each treatment to remove the excess of sterlient.

**Culture media**

Murashige and Skoog’s medium (1962) Gamborg’s (B2) medium (1968) and White’s medium (1943) were used to test the response of explants using different concentrations and combinations of growth regulators. The nutrient medium containing inorganic and organic compounds was stabilized with 0.8% Difco-bacteriological grade agar, pH was adjusted to 5.8 to 5.9. The cultures were incubated at 25 ± 2°C and relative humidity of 60 to 70% under 16 h daily illumination of approximately 1000 to 2000 lux provided by cool fluorescent tubes.

**Response of explants on different types of media**

An initial experiment was conducted to test the response of shoot tip explants on different types of media, such as Murashige and Skoog’s media (1962), B5 medium (Gambrog’s et al., 1968) and White’s medium (1943) for shoot multiplication.

**Response of shoot tip explants on MS medium with different concentrations and combinations of cytokinins**

Shoot tip explants were inoculated on to the MS medium with different concentrations of BAP (0.5 to 4 mg/l) and Kn (0.5 to 4 mg/l) for induction of multiple shoots.

**Root induction**

*In vitro* developed shoots were transferred to half strength MS liquid medium with different concentrations of IBA (1 to 3 mg/l) or NAA (1 to 3 mg/l) for root induction. For this, the shoots of 1.5 to 2.5 cm long with 3 to 4 completely formed leaves were selected and placed on the liquid medium with Paper Bridge in such a way that only shoot base was in touch with the medium. Incubation conditions were similar to them for shoot cultures.

**Acclimatization**

The well rooted plantlets were transferred to sand:soil (1 : 1) in plastic pots and covered with polythene bags having small holes and maintained under culture room temperature 22 ± 2°C with 80 to 90% of relative humidity for one week. The pots were irrigated with quarter strength of MS basal salt solution and when planted lets produced new leaves they were transferred from culture room to greenhouse and eventually to field conditions. Survival percentage was recorded for four weeks transferring to open field.

**Antioxidant activity: Field grown plants V/S in vitro propagated plants**

**Antioxidant activity of NLE and ILE**

Samples preparation: Aqueous extracts were prepared by boiling 1 g of dried natural leaves and *in vitro* propagated leaves powder in 10 ml water, cooled, centrifuged at 1500 g and the supernatant was used for antioxidant activity determination as per Duh and Yen (1997) method.

**Reducing power ability**

Reducing power of water extracts was determined according to the method of Gülčin (2006a, b). Water extracts constituting (5 to 15 mg) were mixed with 5.0 ml of phosphate buffer (0.2 mM, pH 6.6) and potassium ferricyanide (5.0 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (5.0 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 2800 g for 10 min. The upper layer of the solution (5 ml) was mixed with distilled water (5 ml) and ferric chloride (1 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

**DPPH radical scavenging activity**

The effect of herbal water extracts on DPPH radical was estimated according to the method of Duh and Yen (1997). The extracts (50 to 250 µg) were added to a methanolic solution (1 ml) of DPPH radical (final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and left to stand at room temperature for 30 min. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm.
Determination of total phenolics

The phenolic content was determined colorimetrically using Folin-Ciocalteau (FC) method of Gulcin et al. (2004a, b). A sample aliquot of 100 µl was added to 900 µl of water, 5 ml of 0.1 N FC reagent and 4 ml of saturated sodium carbonate solution (75 g/100 ml) and mixed in cyclomixer. The absorbance was measured at 765 nm with Shimadzu UV-visible spectrophotometer after incubation for 2 h at room temperature. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram sample.

Statistical analyses

Antioxidant activity test was carried out in triplicates and the results were recorded as mean ± SD (n=3). Tissue culture data in the figures are given as mean ± SD (n=25). Statistical significance was examined through one-way analysis of variance and Duncan multiple range test. Significant differences were accepted at P < 0.05.

RESULTS

A preliminary experiment was conducted to test the response of shoot tip explants on different types of media, viz., MS (Murashige and Skoog’s, 1982) medium, White’s (1943) medium, B₅ medium (Gamborg’s et al., 1968) with BAP (1 to 3 mg/l) for shoot bud multiplication. It was observed that shoot tip explants on MS medium showed better response than on B₅ and White’s media respectively (Figure 1).

For further experiment were conducted using MS medium fortified with different concentrations and combinations of cytokinins (BAP and Kn) for multiple shoot bud induction. The results are presented in the Table 1. The explants started to elongate within 2 weeks of incubation on basal medium and ended up producing only a few shoot buds (1 to 2). However, on MS medium with different concentrations of BAP (0.5 to 4 mg/l), multiple shoot bud formation was observed. Highest number of shoots buds produced on medium with 2 mg/l of BAP within 3 weeks of incubation (Plate 1A). After 4 weeks of culture, the shoot buds well expanded with leaves. Whereas, on MS medium with other cytokinins, that is Kn (0.5 to 4 mg/l) also produced multiple shoot buds, but the shoot buds were fewer than medium containing BAP and shoot were stunted in growth (Plate 1B). Where as, when both the cytokinins that is, BAP (0.5 to 4 mg/l) and Kn (0.5 to 4 mg/l) were supplemented in combination, less number of shoot buds were formed than the individual concentrations of BAP and Kn (Plate 1C). The present investigation revealed that different levels of BAP were most effective for induction of multiple shoots.

Whereas medium with Kn alone and also in various combinations with BAP was found to be less effective for induction of multiple shoot buds.
Table 1. Effect of different concentration and combinations of BAP and Kn on shoot bud multiplication from shoot tip explants of *M. nudicaulis*.

<table>
<thead>
<tr>
<th>MS + growth regulators (mg/l)</th>
<th>Number of shoots M ± S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.20 ± 0.02</td>
</tr>
<tr>
<td>BAP</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>05.60 ± 0.11</td>
</tr>
<tr>
<td>1</td>
<td>07.60 ± 0.11</td>
</tr>
<tr>
<td>2</td>
<td>20.30 ± 0.10</td>
</tr>
<tr>
<td>3</td>
<td>16.75 ± 0.14</td>
</tr>
<tr>
<td>4</td>
<td>12.00 ± 0.14</td>
</tr>
<tr>
<td>Kn</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>02.50 ± 0.11</td>
</tr>
<tr>
<td>1</td>
<td>07.95 ± 0.13</td>
</tr>
<tr>
<td>2</td>
<td>12.00 ± 0.50</td>
</tr>
<tr>
<td>3</td>
<td>09.55 ± 0.50</td>
</tr>
<tr>
<td>4</td>
<td>08.90 ± 0.45</td>
</tr>
<tr>
<td>BAP+Kn</td>
<td></td>
</tr>
<tr>
<td>0.5 + 0.5</td>
<td>02.90 ± 0.02</td>
</tr>
<tr>
<td>1 + 1</td>
<td>08.00 ± 0.00</td>
</tr>
<tr>
<td>2 + 2</td>
<td>10.02 ± 0.00</td>
</tr>
<tr>
<td>3 + 3</td>
<td>07.80 ± 0.00</td>
</tr>
<tr>
<td>4 + 4</td>
<td>07.00 ± 0.12</td>
</tr>
</tbody>
</table>

Data given are Mean of triplicates ± standard error, values followed by superscript letters through columns differs significantly at (P < 0.001) 1% level when subjected DMRT.

Root induction

Each *in vitro* regenerated shoot buds were transferred to MS half strength liquid medium with different concentrations and combinations of IBA and NAA. At IBA (2 mg/l) produced healthy sturdy root. NAA at 2 mg/l, also produced root but it was very weak.

Acclimatization

The well-established plant lets were transferred to pots containing sand and soil (1:1) and irrigated with MS basal salt solution (quarter strength) for one week maintained at room temperature 22 ± 2ºC with relative humidity 80 to 90% plant lets shows healthy growth and vigor. When they were gradually transferred from culture room condition to green house and eventually to field condition, survivability rate was 90% (Plate 1D).

Determination of antioxidant activity

Extracts with phenolic content between 0.05 to 1.25 mg were evaluated for antioxidant activity in both native and *in vitro* derived leaves of *M. nudicaulis*. Results presented in Figures 2 and 3 indicated dose dependent increase in activity, with a consistent 2.4 and 1.3 fold increased antioxidant activity in *in vitro* derived samples in both reducing power (Figure 2) and free radical scavenging activity respectively (Figure 4).

In addition we examined the content of total phenolics in NLE and ILE by FC method. An approximate 1.75 fold increased total phenolic content was observed in *in vitro* derived leaves (2.502 mg/g) than that of NLE (1.43 mg/g).

This increased phenolic content in ILE indicated a good correlation between the levels of phenol and antioxidant activity. Antioxidant of *M. nudicaulis* can therefore be polyphenolic in nature.

**DISCUSSION**

In India the Ayurvedic system of medicines has been in use for over three thousand years. Charaka and Susruta, developed samhitas based on herbal sources and is still esteemed even in this day as treasure of indigenous medicines. These indigenous medicines are preferred over allopathic medicines since the latter causes lot of side effects due to its synthetic nature. The choice of today’s therapy is therefore exploration of plant drugs. However, due to the overexploitation of the medicinal plants, many of them have become endangered. Plant biotechnology has played an important role in the mass multiplication for conservation and also to procure
innumerable content of drugs.

Cancer is the major killer disease in most developed as well as developing countries, which is induced by oxidative stress (Bandopadhay, 2001; Gulcin, 2009). Hence antioxidants which are very effective in combating cancer needs thorough search especially safer compounds from plant sources. Increased oxidative stress encountered in body due to either environmental hazard, or impairment in the body metabolism due to varying disease conditions including drugs or having insufficient amount of dietary antioxidants, has to be curbed by exogenous supply of antioxidants as a
Figure 2. Determination of antioxidant activity by reducing power assay in *M. nudicaulis*.

Figure 3. Reducing power assays of various amounts of water extracts of *M. nudicaulis*. 
choice of therapy or preventive measure. Natural antioxidants are preferred over that of allopathic drugs to overcome the side effects. Antioxidants thus reported from medicinal plants play an important role as a health beneficiary component. Further, ingestion of the crude plant extract as described in ayurveda medicine may also cause toxicity and hence one has to limit doses. Under such circumstances, antioxidant enriched fraction. The present study also clearly demonstrated that among different types of nutrient media such as MS, B5, White’s media, MS media was found more effective for multiple shoot induction of *M. nudicaulis* than other types of media, as has been reported by earlier reporter (Jyothi and Dhar, 1996). In the present investigation demonstrated that the shoot tip culture of *M. nudicaulis* was found to be suitable material for multiple shoot induction on different concentrations and combinations of BAP and Kn. BAP at 2 mg/l was found to be best for multiple shoot induction. The stimulating effect of BAP on bud breaks and multiple shoot formation has been reported earlier for several medicinal and aromatic plant species including *Ocimum spp* (Pattnaik and Chandra, 1996) and in *Vitex negundo* (Das et al., 2005).

On the other hand Kn was not effective in induction of multiple shoot buds as has been reported by Chandra and Nagesh (1997). There have been several reports of tissue culture yielding enhanced level of secondary metabolites than that of the native plant. It has also been established that slow growing callus accumulates secondary products (Tejavathi and Rao, 1998; Dias et al., 2000). However, very scanty reports are available regarding the validation of *in vitro* callus for bioactivity. In the current study, attempts were made to analyze antioxidant levels in *in vitro* propagated plant compared to that of native plant.

The electron donating capability of rhizome callus and native rhizome extract as evaluated by the reduction of Ferric chloride and potassium ferricyanide complex showed the greatest reducing power with dose dependency in *in vitro* propagated leaves compared to native leaves extract. Therefore both the extracts were electron donors especially the *in vitro* propagated leaves extract and can react with free radicals to convert them to more stable products and terminate radical chain reaction.

DPPH, which is a stable free radical been used to evaluate the antioxidant activity of plant extracts. In the present study, DPPH scavenging activity was found in both *in vitro* propagated leaves and native leaves extract. Comparatively *in vitro* propagated leaves extract exhibited a higher antioxidant activity. In addition, the ability to scavenge the DPPH radical is related to the inhibition of lipidperoxidation (Kaur and Perkins, 1991). Phenolic compounds are commonly encountered in the plant kingdom, and they have been reported to have multiple biological effects, including antioxidant activity (Halliwell and Gutteridge, 1981; Duh and Yen, 1997). In the current study, we examined the phenolic content of

![In vitro derived leaf extracts](image)

**Figure 4.** DDPH scavenging activity of native leaf and *in vitro* propagated leaves extracts of *M. nudicaulis*. 

0.05 0.1 0.15 0.2 0.25

% DPPH radical scavenging activity

Amount of extract (mg)
in vitro propagated leaves and native leaves extract. Our results showed that in vitro propagated leaves exhibited a higher total phenolic content than the native leaves extract based on the Folin-Ciocalteau method. The antioxidant activity of both the extracts showed a dose dependent response in terms of phenolic content. Even the higher antioxidant activity of in vitro propagated leaves correlated with the increased total phenolic content. The results clearly demonstrated that the antioxidant activity of both the extracts and the higher activity in the in vitro propagated leaves may be attributed to the total phenolic content.

REFERENCES

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