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In vitro Studies on *Delonix elata* L. - An Endangered Medicinal Plant

Ghada Abd El-Moneim Hegazi

Tissue Culture Unit, Plant Genetic Resources Department,
Desert Research Center, El-Matarya, Cairo, Egypt, P.O. Box 11753

Abstract: Investigations were carried out to determine antioxidant properties and total phenol content of three different explants from *in vitro* germinated seedlings of *Delonix elata* L. and their respective callus cultures. High efficient callus was induced from leaves, stems and roots of *D. elata* seedlings on Murashige and Skoog (MS) medium containing 0.5 mg/l β-Naphthalene acetic acid (NAA) and 6-benzyladenine (BA) or Kinetin (Kn). Callus induction rate reached 100% in all tested explants. All extracts had antioxidant activities, but those of stems had the highest values (1.18 and 0.26 mmol eq. Trolox/g fresh weight of sample for the stems and their callus, respectively). All the explants and their callus displayed phenol contents and also those of stems had the highest amounts (27.52 and 6.670 mg/g fresh weight of sample for the stems and their callus, respectively). A positive correlation between trolox equivalent antioxidant capacity (TEAC) values and total phenol content was found, which showed that phenolic compounds largely contribute to the antioxidant activity of this plant.

Key words: *Delonix elata* · *In vitro* germination · Callus · Antioxidant properties · Phenols

INTRODUCTION

Legumes have been the focal point for biological interest, not only because of their protein rich seeds and ability to fix atmospheric nitrogen, but also for their importance as a source of timber and fuel wood [1]. *Delonix elata* is an endangered deciduous tree from family Fabaceae (Leguminosae), subfamily Caesalpinioideae. It is endemic to Egypt. The tree is under immediate threat as a source of timber and overgrazing is another causative for its decline [2]. *D. elata* is a multipurpose tree. It is a promising source of micronutrients for goats, sheep, camels and cattle, which eat the foliage and young pods. It is very promising as a firewood source. The tree yields a dark coloured, mucilaginous gum. *D. elata* has a potential use in soil erosion control. Also, it is a good tree for reforestation of difficult sites. The medical usefulness of the tree is acknowledged, the decoction of the leaves are used to get relief from rheumatic problems like pain and stiffness of the joints, especially the knees. The root decoction is drunk for abdominal pains. Seed oil of *D. elata* contains small amounts of sterculic and malvalic acids [3]. *D. elata* leaves contains high levels of phenolic compounds and mould [4]. Also, Goromela *et al.* [5] indicated that irrespective high concentration of phenolic compounds-amyrin, hesperitin and neohesperidin were isolated from the dried roots of *D. elata*.

Plant derived polyphenols are of great importance because of their potential antioxidant properties. Phenolic compounds exhibit a considerable free-radical scavenging (antioxidant) activity, which is determined by their reactivity as hydrogen-or electron-donating agents, the stability of the resulting antioxidants-derived radicals, their reactivity with other antioxidants and finally their metal chelating properties [6]. Free radicals are produced as a part of normal metabolic processes. They are extremely reactive, highly unstable and potentially damaging transient chemical species. Under physiological conditions, the cellular redox state is tightly controlled by antioxidant enzymatic systems and chemical scavengers such as endogenous enzymes, dietary antioxidants as well as some hormones [7]. However, an over-production of free radicals on one side and/or deficiency of antioxidants on the other, will lead to a significant increase in the production of these radicals, which overwhelm the antioxidant defense and impose oxidative stress on the physiological system [8]. The excess oxidative stress can cause damage to cellular lipids, proteins, or DNA, inhibiting their normal functions. Oxidative stress has

Corresponding Author: Ghada Abd El-Moneim Hegazi, Tissue Culture Unit, Plant Genetic Resources Department, Desert Research Center, El-Matarya, Cairo, Egypt, P.O. Box 11753.

been implicated in many degenerative diseases such as atherosclerosis, coronary heart diseases, aging and cancer [9]. Therefore, consumption of antioxidant constituents reported to have protection against oxidative damage, in which free radicals are involved, that induced degenerative and pathological processes including ageing and cancer [10]. In addition, antioxidants have been widely used in the food industry to prolong the shelf life. Nowadays, natural antioxidants, due to their limited sources and high price, are not widely used. Synthetic antioxidants, such as butylated hydroxytoluene and butylated hydroxyanisole are commonly used in the food industry. However, there is a widespread agreement that synthetic antioxidants need to be replaced with natural antioxidants because some synthetic antioxidants have shown potential health risks and toxicity, most notably possible carcinogenic effects [11]. Therefore, it is great of importance to find new sources of safe and inexpensive antioxidants of natural origin in order to use them in foods and pharmaceuticals preparations to replace synthetic antioxidants. Antioxidant properties have been studied in several plant species for the development of natural antioxidant formulations in the areas of food, medicine and cosmetics [12]. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world due to their potential antioxidant activities in reducing free radical induced tissue injury, no side effects and economic viability [13].

The continuously increasing demands for therapeutic molecules, produced by ever greener processes, together with concerns regarding sharp current reduction in biodiversity (and associated pressures to avoid collecting rare plants from the wild and/or using scarce habitats to cultivate them), are driving efforts to find alternative ways to produce high value plant-derived metabolites [14]. Partly for these reasons, plant callus culture technology is becoming an increasingly attractive and cost-effective alternative to classical approaches for the mass production of plant-derived metabolites. Indeed, it is the only economically feasible way of producing some high-value metabolites from difficult-to-access, rare and/or threatened plants [15]. So far, there is no report about the tissue culture and callus culture about of *D. elata* in the literature.

The present study involved a comparison between three explants of *D. elata,* from *in vitro* germinated seedlings and their callus cultures due to their potential to produce antioxidant activity in relation to total content of phenolic compounds.

MATERIALS AND METHODS

Plant Materials and Culture Conditions: Seeds of *D. elata* were collected from Gabal Elba, South Egypt. They were first placed under running tap water for 30 minutes. Before surface sterilization, a section of the seeds were immersed in 100 mg/l gibberellic acid (GA_3; Sigma Cell Culture, min. 90%, St. Louis, USA) for 1 day. In a transfer hood, seeds were soaked in a 50% (v/v) Clorox bleach solution (2.5% sodium hypochlorite) for 30 minutes, providing gentle agitation, followed by three sequential rinses for 1 minute in sterile distilled water. Ethyl alcohol at a concentration of 95% was poured into a small beaker. A second section of the sterilized seeds were placed into the beaker for approximately 1 minute. With forceps, seeds were extracted one by one at a time and passed briefly (2 to 3 seconds) through a flame. The third section of sterilized seeds was remained as a control without any pre-treatments. All seeds were placed directly on the surface of full-strength Murashige and Skoog (MS) [16] medium (Duchefa, Haarlem, the Netherlands) supplemented with 30 g/l sucrose. The pH of the medium was adjusted to 5.7 to 5.8 before being solidified with 3 mg/l phytagel (Duchefa, Haarlem, the Netherlands), then autoclaved at a pressure of 1.06 Kg/cm^2 and 121°C for 15 minutes. The cultures were incubated at approximately 25±2°C with a 16-hours photoperiod under cool white florescent tubes (F140t9d/38, Toshiba). Four-week-old *in vitro* germinated seedlings of *D. elata* were used as a source of explants for initiation of callus cultures.

Callus Induction and Subculture: Leaves, stems and roots were excised from the seedlings and cut into small pieces (0.5-1 cm), then cultured aseptically on solid MS medium supplemented with various plant growth regulators (PGRs) (Sigma Cell Culture, min. 90%, St. Louis, USA), such as β-Naphthalene acetic acid (NAA) (0.5-1 mg/l) as an auxin and 6-bezylaminopurine (BA) or Kinetin (Kn) (0.5-1 mg/l) as cytokinins. In addition to MS medium without PGRs. Each experiment contained at least 40 replicates and was repeated twice. Data were recorded after four weeks of culture. Well grown callus induced from explants were selected to subculture to the same medium composition after every four weeks. The best PGRs ranges were chosen for every explant type after the third subculture based on the callus observation and fresh weight. After a period of three-subcultures, established callus cultures were taken up for growth study and quantification of antioxidant activity and total phenols.

Determination of Total Antioxidant Activity: Free radical scavenging activity of plant samples against stable 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH, Sigma-Aldrich Chemie, Steinheim, Germany) was determined spectrophotometrically. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced.

The change in colour (from deep-violet to light-yellow) was measured at 515 nm using Shimadzu Spectrophotometer (UV-1601 PC) (Shimadzu, Kyoto, Japan). Radical scavenging activity of plants was measured by method of Brand-Williams *et al.* [17]. Extracts were prepared by dissolving a known weight of plant sample in 10 ml of methanol (HPLC grade). The solution of DPPH in methanol ($6X10^{-5}$ M) was prepared freshly, before UV measurements. Twenty nine ml of this solution were mixed with 100, 300 and 500 µl of plant extract in 1 cm path length cuvette. The samples were kept in the dark for 15 minutes at room temperature and then the decrease in absorbance was measured. The reference cuvette contained DPPH blank.

The radical scavenging activity of the samples was expressed as percent inhibition of DPPH radical as following:

$$\text{Inhibition\%} = [(A_{control}-A_{sample})/ A_{control}] \times 100$$

Where: $A_{control}$ is the absorbance of the control (the blank solution without plant extract) and A_{sample} is the absorbance of the sample.

Finally, the antioxidant capacity of the samples were compared to that of a synthetic antioxidant; 6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox; Sigma-Aldrich Chemie, Steinheim, Germany) and expressed as Trolox equivalent antioxidant capacity values (TEAC). IC_{50} values, indicating concentration of sample required to inhibit DPPH radical formation by 50%, were then calculated.

Determination of Total Phenolic Content: The Folin-Ciocalteau method (FCM) in triplicate was conducted for determination of total phenolic compounds. It based on the reduction of a phosphowolframate-phosphomolybdate complex (Folin reagent, Sigma-Aldrich Chemie, Steinheim, Germany) by phenolics to blue reaction products, as described by Singleton *et al.* [18]. Plant samples (known weight) were extracted at room temperature and in the absence of light with methanol (HPLC grade) containing 1% butilated hydroxyl toluene (BHT, Sigma-Aldrich Chemie, Steinheim, Germany) and the resultant mixture was left at room temperature for 1 hour and then filtered through Whatman no. 4 filter paper. Briefly, 0.1 ml of each extract was mixed with 2.5 ml of 1:10 diluted Folin-Ciocalteu's phenol reagent, followed by 2ml of 20% (w/v) sodium carbonate. After 5 minutes at 50°C, absorbance was measured at 760 nm using Shimadzu Spectrophotometer (UV-1601 PC) (Shimadzu, Kyoto, Japan). The results were expressed as gallic acid equivalent (GAE). Calibration curve was carried out with gallic acid (Sigma-Aldrich Chemie, Steinheim, Germany) methanolic solutions (0.5-7 µg/ml) and values were presented as the mean of triple analyses. Phenol content of the extracts was expressed as mg/g fresh weight of sample.

Statistical Analysis: The experiments were subjected to the completely randomized design. Variance analysis of data was carried out using ANOVA program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan's multiple range test [19].

RESULTS AND DISCUSSION

***In vitro* Seed Germination:** In family Fabaceae; the fruit is usually a legume and typically possess hard, smooth seeds. Physical dormancy is present, caused by an impervious seed coat that requires scarification to initiate the germination process [20]. Therefore, two pre-treatments were applied to enhance the germination of *D. elata*; application of GA_3 and flaming of seeds that are known to break seeds dormancy, in addition to the control (without any pre-treatments), as it is an endangered plant in Egypt and very few amounts of seeds could be obtained. Comparing the two pre-treatments for the *in vitro* germination of the seeds of *D. elata* (Table 1), it is noticed that GA_3, which is known to promote seed germination by the induction of the synthesis of a number of hydrolyses that mobilize reserve materials in the seeds, did not enhance the survival or germination percentages of seeds if compared with the control.

On the other hand, the survival percentage increased significantly with the flaming of seeds, which helped in their effective surface sterilization. Also, by using the flaming technique the percentage of seed germination reached its highest value of 66.41% comparing to the other treatments. Within one week, germination was visible and an average of 6.5-cm long seedling was

Table 1: Effect of different pre-treatments on the survival and *in vitro* germination percentages of *D. elata* seeds

Treatments	Survival %	Germination %
Control (without pre-treatments)	91.67[b]	51.53[b]
GA_3 treatment	91.33[b]	45.00[b]
Flaming of seeds	99.30[a]	66.41[a]

Fig. 1: *D. elata* seedlings about 4 weeks after germination *in vitro*.

obtained in just 3 weeks (Fig.1). One main advantage of this technique is that it saves time-without flaming, these seeds normally take at least 6 weeks to germinate. Sugii [21] reported that soaking Fabaceae seeds in alcohol and then passing them through a flame for 2 to 3 seconds and allowing the alcohol to burn are a quick and easy way of promoting germination of at least 8 species of endemic Hawaiian legumes. In addition to stimulating germination, the technique effectively sterilizes seeds for *in vitro* germination. Also, at the Lyon Arboretum, scientists work with very small amounts of seeds and flaming is apparently effective in weakening the hilum, which then allows water imbibitions to occur.

Callus Induction and Subculture: Previous primary experiments on the callus induction from different seedling's explants of *D. elata* indicated that NAA in combination with BA or Kn in the medium was the most suitable for callus induction and subculture. This confirms that different types of auxins had various effects [22, 23]. The callus induction rate was recorded maximum (100%) for all explants on the different tested media containing NAA. Depending on the chosen cytokinins, Kn and BA, the responses to increase callus fresh weight varied among treatments. The supplement of BA in combination with NAA in the medium was found more effective in comparison with Kn in increasing the fresh weight of callus in leaves. While Kn was more suitable in increasing the fresh weight of callus induced from stems and both cytokinins produced callus effectively from roots. This finding is in harmony with those obtained by Rao *et al.* [24] who mentioned that the cytokinins facilities the effect of auxin in callus induction.

The callus growing state varied with the explants type as; the callus of leaves was green, compact, hard and granular (Fig. 2a), while that of stems was yellow, loose and spongy (Fig.2b) and that of roots was brown and granular (Fig. 2c). The PGRs combinations that were more effective for each explant are represented in Table 2 and were; MS medium free from PGRs, 0.5 mg/l NAA in addition to 0.5 or 1 mg/l BA for leaves, 0.5 mg/l NAA in addition to 0.5 or 1 mg/l Kn for stems and 0.5 mg/l NAA in addition to 0.5 mg/l BA or Kn for roots. Among the tested explants; leaves recorded 3.051 g fresh weight of callus/explant and thus achieved the highest callus amount. It is a known fact that callus growth and development are strongly related to genetic background and physiological status, the source of tissue, chemical compositions and physical state of the culture medium and culture conditions [25]. Besides the genetic potential of the donor plant for callus induction and the growth of callus in an *in vitro* culture, the choice of suitable plant growth regulators and the medium is always considered to be as the most important step. MS medium is one of the

(a) (b) (c)

Fig. 2: Callus of *D. elata*, (a) Callus from seedling's leaves, (b) Callus from seedling's stems and (c) Callus from seedling's roots.

Table 2: Effect of MS medium containing chosen PGRs ranges on callus fresh weight and growing state of callus

Variants	Average final fresh weight of callus (g/explant)	Growing state of callus
Callus from leaves on:		
PGRs free medium	1.959^{b}	Green, compact, hard and granular
0.5 mg/l NAA+0.5 mg/l BA	1.610^{c}	
0.5 mg/l NAA+1 mg/l BA	3.051^{a}	
Callus from stems on:		
0.5 mg/l NAA+0.5 mg/l Kn	1.055^{d}	Yellow, loose and spongy
0.5 mg/l NAA+1 mg/l Kn	1.443^{cd}	
Callus from roots on:		
Callus on 0.5 mg/l NAA+0.5 mg/l BA	1.045^{d}	Brown and granular
Callus on 0.5 mg/l NAA+0.5 mg/l Kn	1.411^{cd}	

Table 3: A comparison of antioxidant activity and total phenol content in three explants of *D. elata* seedlings and their callus.

Sample	TEAC* (mmol eq. Trolox/g fresh wt.)	IC_{50} (g)	Total phenols (mg/g fresh wt.)
Leaves	0.19	0.023	14.65^{c}
Callus on PGRs free medium	0.04	0.179	3.440^{g}
Callus on 0.5 mg/l NAA+0.5 mg/l BA	0.19	0.064	5.300^{e}
Callus on 0.5 mg/l NAA+1 mg/l BA	0.23	0.034	6.160^{d}
Stems	1.18	0.002	27.52^{a}
Callus on 0.5 mg/l NAA+0.5 mg/l Kn	0.04	0.085	2.590^{h}
Callus on 0.5 mg/l NAA+1 mg/l Kn	0.26	0.057	6.670^{d}
Roots	1.16	0.009	22.63^{b}
Callus on 0.5 mg/l NAA+0.5 mg/l BA	0.11	0.128	3.840^{fg}
Callus on 0.5 mg/l NAA+0.5 mg/l Kn	0.15	0.048	4.420^{f}

*TEAC: Trolox equivalent antioxidant capacity

most preferred medium for callus induction and callus growth because of its sufficient nutrients [26].

Antioxidant Activity: DPPH stable free radical method is a relatively rapid and sensitive way, which when encounters proton donors; such as antioxidants, the radicals get quenched and absorbance gets reduced and thus used to survey the antioxidant activity of a specific compound or plant extracts [27]. The antioxidant tests based on DPPH radical scavenging capacity assay are presented in Table 3.

The results revealed that the seedlings and callus had antioxidant activity, but the callus showed lower activity than the seedling's explants. They also indicated that among the three explants of *D. elata* seedlings, the methanolic extracts of stems and roots showed the highest activity compared to the leaves with amounts of 1.18, 1.16 and 0.19 mmol eq. Trolox/g fresh wt., respectively. On the other hand, in case of leaves, the antioxidant activity of the callus produced on the medium containing 0.5 mg/l NAA and 1 mg/l BA gave a higher value (0.23 mmol eq. Trolox/g fresh wt.) than that of leaves of the seedlings (0.19 mmol eq. Trolox/g fresh wt.) and the callus produced on the medium containing 0.5 mg/l of each of NAA and BA gave the same value as the leaves. Antioxidants are broadly defined as molecules that when

present at low concentrations, compared to those of oxidizable substrates, significantly delay or prevent oxidation of that substrate. The antioxidant potential of *D. elata* extracts seems to be due to its strong hydrogen-denoting and metal chelating ability, as well as to its effectiveness as a scavenger of hydrogen peroxide and free radicals.

Based on the DPPH scavenging activity assay results, Khorasani *et al.* [28] also found that the highest scavenging capacity of *Asparagus officinalis* cv. Mary Washington was obtained from *in vivo* plant extract followed by *in vitro* plant extract, while there was no significant difference between these two samples and callus extract showed significantly lower scavenging potential when compared to the other plant extracts. The results also confirm the study of Tanwer *et al.* [29] who showed that antioxidant activity of methanolic extract of the *in vivo* grown *Spilanthes acemella* is higher than the *in vitro* grown callus. The concentration for 50% inhibition (IC_{50}) of the plant extracts examined in this study for scavenging effects on DPPH free radical is shown also in Tables 3. The IC_{50} value decreased with the increase of antioxidant activity of each explant type and *vice versa*.

Phenols Content: Phenolic compounds are a class of antioxidant agents which act as free radical terminators [30]. Based on the absorbance values after reaction with Folin-Ciocalteu reagent, results of the spectrophotometric analysis are given in Table 3. The amount of total phenols was higher in seedling's explants and lower amount was recorded in their respective callus. Similar results were also observed by Tanwer *et al.* [29] in *Spilanthes acemella* Murr. and Sagwan *et al.* [31] in *Pongamia pinnata* L. Pierre. The stems contained the highest amount of phenol of 27.52 mg/g fresh wt. and its callus achieved the highest amount of 6.67 mg/g fresh wt. too (if compared to the other callus tissues), on the MS medium containing 0.5 mg/l NAA and 1 mg/l Kn. It could be noticed that the samples that had the highest phenolic content had also the highest antioxidant activity and the lowest IC_{50} value, which ensure that the phenolic compounds in *D. elata* are responsible for its antioxidant activity. As the strong correlations between the results, they showed that phenol compounds largely contribute to the antioxidant activity of this plant. Many studies have demonstrated the radical scavenging properties of plant phenolic compounds and confirm the relationship between phenolic compounds and antioxidant activity [32].

The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donars and singlet oxygen quenchers [33]. According to the present study, the high contents of phenol in *D. elata* seedlings can explain its high free radical scavenging activity. Also, Bendini *et al.* [34], Dlugosz *et al.* [35] and Wojdylo *et al.* [6] found that there was a strong relationship between total phenol content and antioxidant activity, as phenols are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolic content of plants may be contributed directly to their antioxidant action.

CONCLUSION

The results of the present study showed that the extract of *D. elata* seedling's explants and their respective callus had the potential to induce antioxidant activity and phenolic metabolites, but it seemed that the undifferentiation of cells in callus cultures could be a reason for expressing these desired secondary metabolites in lower ranges. Nevertheless, this work provides basic information for massive production of phenolic compounds as antioxidants in *D. elata* and indicated the ability to utilize plant biotechnology techniques towards development of desired bioactive metabolites in *in vitro* culture as an alternative way to avoid using endangered or rare wild plants in pharmaceutical purposes.

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