South African Journal of Botany 108 (2017) 149–156

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

In vitro propagation from bud and apex explants of *Moringa oleifera* and evaluation of the genetic stability with RAMP marker

J.A. Avila-Treviño [a], J.M. Muñoz-Alemán [a], E. Pérez-Molphe-Balch [a], A. Rodríguez-Sahagún [b], J.F. Morales-Domínguez [a,*]

[a] Universidad Autónoma de Aguascalientes, Centro de Ciencias Básicas, Departamento de Química, Laboratorio de Biología Molecular de Plantas, edificio # 60 C.U. Av. Universidad # 940 Fracc. C.U., C.P. 20131 Aguascalientes, Ags., México

[b] Universidad de Guadalajara, Centro Universitario de la Ciénega, Av. Universidad N° 1115, Col. Lindavista, C.P. 47820 Ocotlán, Jalisco, México

ARTICLE INFO

Article history:
Received 29 February 2016
Received in revised form 29 June 2016
Accepted 2 October 2016
Available online xxxx

Edited by J Van Staden

Keywords:
Medicinal plant
Somaclonal variation
Regeneration
Explant

ABSTRACT

Moringa oleifera is the best known species of the Moringaceae family attributed with several medicinal uses and a high nutritional value. This plant is native of the western sub-Himalayan and now is distributed worldwide. The conventional way to propagate Moringa is by seeds, causing its growth to be slow. An alternative to propagate plants in less time and greater number is through plant tissue culture (PTC), which broadly refers to cultivation of plant cells, tissue and organs on artificial medium under aseptic and controlled environmental conditions. Therefore, in this study, an effective system for mass propagation of *M. oleifera* and the genetic stability with RAMP marker has been implemented. For the propagation, explant of buds and apices of the cotyledon node was used in MS medium and without Plant Growth Regulators (PGR). During indirect regeneration, the best treatment corresponded to the combination of 1 mg L^{-1} BA and 0.2 mg L^{-1} AIA, which had a maximum of 14 buds per explant; with the same treatment, but using leaves as explants there was mass production of roots. Acclimatization and ground transference of plants into the soil had 95% survival average. DNA from leaves propagated and regenerated and *ex vitro* plants were used to study genetic variability through Random Amplified Microsatellite Polymorphism (RAMP); the dendrogram of this assay did not show any significant variation between the plants. The *in vitro* propagation protocols developed here would be useful to obtain mass plants in less time and enhance propagation.

1. Introduction

Moringa oleifera Lam. is the best known species of the Moringaceae family. This plant is native of the western sub-Himalayan, India, Pakistan, Asia and Africa, and now is distributed in the Philippines, Cambodia, America, and the Caribbean Islands (Luqman et al., 2012). In Mexico it is mainly introduced throughout the Pacific and Gulf of Mexico (Mohammed et al., 2012), and has been shown to have high adaptation in tropical areas while in extreme climate areas its growth is slow. *M. oleifera* is credited with various medicinal properties; for example, the leaves have a high nutritional value and are an important source of highly digestible protein, calcium, iron, vitamin C, and antioxidants such as flavonoids, phenols, carotenoids and vitamin E (Park et al., 2011; Luqman et al., 2012; Förster et al., 2013) and the seeds are considered as an effective natural coagulant used to purify water, and as a potential source of biodiesel. Most of the plant has been used as a traditional medicine for the treatment of a variety of disorders such as: respiratory diseases, hypertension, diabetes, anemia and cancer (Park et al., 2011).

The conventional way to propagate *M. oleifera* is by seeds, causing its growth to be slow (Stephenson and Fahey, 2004). Thus, at present with these results it is impossible to cover the growing demands required by this species in order to meet different domestic, nutritional, commercial and medicinal uses (Luqman et al., 2012). A strategy to solve this kind of problems is through Plant Biotechnology, which is based in plant tissue culture (PTC) set of techniques that allows the establishment, maintenance and development of any part of the plant, from an individual cell to a complete plant, under artificial and axenic conditions therefore, the establishment of an *in vitro* tissue culture offers many advantages over the conventional methods of propagation (Yin et al., 2013). In addition tissue culture technique is a powerful tool for the generation of genetically modified plants (Shahzad et al., 2013). There are some *in vitro* propagation studies for Moringa from different explants such as: nodal segments (Förster et al., 2013; Saini et al., 2013), indirect organogenesis (Mathur et al., 2014), multiplication using immature seeds (Stephenson and Fahey, 2004) and regeneration of axillary cotyledons and buds (Steinitz et al., 2009).

* Corresponding author.
E-mail addresses: jaat_13@hotmail.com (J.A. Avila-Treviño), muajazmin@hotmail.com (J.M. Muñoz-Alemán), eperezmb@correo.uaa.mx (E. Pérez-Molphe-Balch), aracelicrs@gmail.com (A. Rodríguez-Sahagún), jfmoral@correo.uaa.mx (J.F. Morales-Domínguez).

http://dx.doi.org/10.1016/j.sajb.2016.10.003
0254-6299/

Among the different paths of *in vitro* cultivation, some of the conditions can be modified to obtain better result. Therefore, changes in these conditions, such as phytohormone composition and concentration or light intensity and temperature, can produce more vigorous plants (Khateeb et al., 2013). In the case of regeneration, different hormone types and concentrations can help in developing callus, when being subjected to dedifferentiation and differentiation processes, which in turn may result in some genetic variation (Roy et al., 2012). Therefore, an important aspect for the regeneration techniques or clonal propagation in order to be successful is that these plants present uniformity compared to the ones that are conventionally propagated (Zhao et al., 2005).

Numerous techniques have been developed to detect variation due to the different effects that may have on the quality of the regenerated plants; the most efficient techniques for this purpose are those which include the use of DNA molecular markers (Roy et al., 2012). These markers provide better opportunities for genetic characterization, biodiversity studies and genetic variability (Sorkheh et al., 2009). Random amplified polymorphic DNA (RAPD) analysis was developed in 1990 (Williams et al., 1990). Since then, RAPD analysis, combined with other molecular techniques such as internal transcribed spacer (ITS), simple sequence repeat (SSR), inter-simple sequence repeat (ISSR), inter-retrotransposon amplified polymorphism (IRAP), amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP), has been widely used in the various unicellular and multi-cellular organisms across various fields for assessments of genetic diversity and characterization of germplasm, identification and fingerprinting of genotypes, and molecular-assisted breeding in ecological, evolutionary, taxonomical, phylogenic and genetic studies (Long et al., 2015). Within these markers, the RAMP (Random Amplified Microsatellite Polymorphism) technique is included, which combines the advantages of ISSR and RAPD markers and has shown better results in cultivar genealogies, more faithfully than the RAPDs; besides being particularly suitable for plant species with an ambiguous genetic background (Zhang et al., 2005). This technique has already been used to evaluate the genetic diversity in legumes such as: *Cicer arietinum* L., *Phaseolus vulgaris* L., *Pisum sativum* L., *Tamarindus indica* L. (Valadez-Moctezuma et al., 2005), and *Leymus Hochst* among others, and allows to show the individual variation base (Yang et al., 2006).

In the present study, propagation and *in vitro* regeneration of *M. oleifera* were performed, where the plants generated by these processes were transferred into the soil. Subsequently, leaf samples were taken for DNA extraction and to analyze the genetic variability through a RAMP molecular marker.

2. Materials and methods

2.1. Plant material and explant preparation

M. oleifera seeds from trees grown in the Santiago Valley, Guanajuato, Mexico (1) and Tuxtepec, Oaxaca, México (2) were used. Seed coats were removed aseptically and seeds were disinfected in a solution of Hi-cleen plus Tween 20 (Thermo Scientific, USA) for 2 min, followed by three washes with sterile distilled water. Subsequently, they were washed with a 70% ethanol solution for 3 min, and one with a 10% solution of commercial bleach for 15 min. Finally, the seeds were eventually washed three times with sterile distilled water. Seeds were planted aseptically in MS basal medium (Murashige and Skoog, 1962) containing 3% sucrose and 0.7% agar (w/v) 5.7 pH, and incubated at 25 ± 1 °C in the dark until germination; subsequently they were placed at 16/8 h light/darkness period for seedling development.

2.2. Clonal propagation

Two different experiments were conducted in order to evaluate the potential of sprout induction and mass multiplication. In the first experiment, the apical shoots that emerged from seed germination were cut and placed in a 100% MS medium without growth regulators to induce new growth development. From new buds, the axillary nodes were cut and placed in a 100% MS at 25 ± 1 °C, and a photoperiod of 16/8 h light/darkness; this process was performed during four propagation cycles. In the second experiment, the seed was germinated and the seedling tip was cut, which led to the creation of new buds in the cotyledonary node. Subsequently, we waited for each bud to develop at least four axillary nodes, and these were propagated as mentioned above; this process was performed for 12 months.

2.3. Indirect regeneration

Stem and leaf segments generated from the propagation methods were used as explants. Ten different treatments using a 100% MS as basal medium, supplemented with different combinations and concentrations of benzylaminopurine (BA), indoleacetic acid (IAA) and indolebutyric acid (AIB) were prepared (Table 1). For each treatment, the stem segments were placed in vertical and horizontal positions and the leaves in an adaxial form until the appearance of callus and buds. The buds were transferred to a 100% MS without growth regulators for rooting. The plantlets obtained from both clonal propagation and regeneration, rooted in the MS basal medium without growth regulators, so it was not necessary to test treatments for this purpose.

2.4. Acclimatization

Rooted plantlets were separated from the medium and washed with distilled water to remove the debris present on the root. Hardening of the rooted plantlet was done in plastic bags containing autoclaved mixture of peat, fertilizer and lime (Peat moos, PROMIX®). Plants were watered, and then covered with transparent polythene bags. The bags were perforated during the first seven days, and thereafter were removed. This procedure was done inside the growing chamber under the same previously described conditions. Finally, the plantlets were placed under natural conditions until their acclimatization.

2.5. Ex vitro germination

Seeds from the two locations were placed and underwent a pretreatment in distilled water for 24 h; then they were seeded in germination trays with a mixture of peat, fertilizer and lime (Peat moos, PROMIX®).

2.6. Statistical analysis

For multiple propagation, the seed germination percentage and the average of buds per seed were recorded. A completely randomized design was used for indirect regeneration with five repetitions of 20 explants each, shoot average per explant, nodal per explant and explant length and the difference between treatments was evaluated with a Duncan multiple range test, using the statistical package GraphPad Prism 6.0.

Table 1
Treatments for inducing indirect organogenesis.

Treatments	BAP mg L^{-1}	IBA mg L^{-1}	IAA mg L^{-1}
T1	1	0.2	–
T2	1	3	–
T3	1	4	–
T4	3	0.2	–
T5	3	3	–
T6	3	4	–
T7	1	–	0.2
T8	1	–	3
T9	3	–	0.2
T10	3	–	3