



Development of In Vitro Root Culture and miRNAs Analysis for Secondary Metabolites of Native Plants from the Mexican Bajío[†]

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Abstract: Mexico is a megadiverse country, with a high quantity of unique plant species with different uses and applications, such as bactericidal, fungicidal, insecticidal, and, recently, nutrimental. The content of phytochemicals, and the impact of them on animal and human health, has made them a target for biotechnological improvement. In the region Bajío in Mexico, several plants that are associated with ecological, medical and industrial potential have been identified, but also those associated with the traditions. The work in this project includes the development of systems for the culture for the production of secondary metabolites (in vitro root tissue culture) and the miRNA expression analysis, in order to find the molecules that are associated with metabolites production. In this study, we include the following two plants: marigold (*Tagetes erecta*), in which genes associated to lutein production had been identified in flower development; systems for cell culture and plant transformation have been developed, but no systems for in vitro root culture. Up until now, there are not studies related to miRNA expression and association to these molecules to secondary metabolites. In *Heliopsis longipes*, several methodologies had been developed for the isolation of afinin and its uses in agriculture, medicine, and, recently, as analgesic activities in some other metabolites. First, a root tissue culture was established for both of the plants (marigold and *Heliopsis*), using a combination of auxins (2,4-D, IAA, IBA) in a kinetic assay, as the base for manipulation; differences in the root architecture were determined mainly in the time of production and root architecture. In the molecular analysis, four miRNAs were found to be differentially expressed, and associated to secondary metabolites production (miR146, miR164, miR168, miR171). The reordering of miRNAs synthesis and the targets was analyzed, and is associated with the secondary metabolites production, in order to establish a system for the in vitro induction of metabolites.

Keywords: auxins; carotenoids; chilcuague; cempaxúchitl; *Heliopsis longipes*

1. Introduction

Mexico is a megadiverse country, and it contains a great number of plant species associated to the culture. However, there is an unknown that is related to its functional properties (food, medicinal, agricultural). It is necessary to identify the secondary metabolites and their mechanisms of control. The ignorance about the components (the metabolites and its regulation) had been carried on the devaluation of the plants, which cause them to be at the level of endangered or underutilized species. In the species of this work, there are not strategies for metabolite identification and biosynthesis, and no genetic sequences that are related to metabolite production, or their regulation has been isolated.

Marigold (*Tagetes erecta*) has been cultivated since the antique times, mainly as ornamental. The plant is used in religious ceremonies. Also, their uses in the pharmaceutical

area are associated with areas such as antiparasitic, antispasmodic, and disease-fighting [1]. In marigold, the genes that are associated with lutein production have been isolated and characterized [2], and the tissue culture and genetic transformation is outlined in [3]. No data associated with secondary metabolites expression and development are reported yet, and even no reports on root culture exist.

Chilcuague (*Heliopsis longipes*) is an endemic plant from Guanajuato, San Luis Potosi and Queretaro. Alcamides metabolism is associated with different activities, such as antifungal, bactericidal, or plant grown [1,4]. More recently, analgesic activity was reported [5], which makes the plant very attractive in order to search for new metabolites that are different than alcamides. It also called the attention of pharmaceutical companies, in order to exploit the plant resources. It will be important to identify the pathways for the synthesis of these new compounds, and to isolate the genetic sequences that control the biosynthesis of these compounds, their activities, and their possible mechanisms of regulation, including miRNAs.

Further, miRNAs are a class of non-codificant, small RNAs that regulate the gene expression in eukaryotes. They are involved in different plant development processes, different disease response mechanisms, and stress [6]. The miRNAs plays an essential role in post-transcriptional gene regulation, and their targets include transcription factors and other regulatory proteins, with a role in plant growth development [7].

In order to identify the miRNAs function, it is necessary to analyze their expression and their targets, which have a negative correlation. The contundent evidence for miRNA function is the expression in transformed plants, where it is possible to evaluate their effect on specific processes. Recently, miRNAs study has been focused on secondary metabolism, and it was possible to correlate the function of miRNAs to secondary metabolites biosynthesis; the miRNAs induced in the root and flowers of marigold and chilcuague are a good example [8].

In this work, we studied regulatory molecules (miRNAs and their targets), in order to identified the metabolite production mechanisms in an in vitro culture system (root culture) of marigold and chilcuague, for their experimental control.

2. Experiments

2.1. In Vitro Germination

Seeds from marigold and *Heliopsis* were disinfested with absolute ethanol, 20% sodium hypochlorite washed with sterile water and exposed to an antifungal compound (PPM), during 12 h, then washed and transferred in sterile conditions to MS at 25 °C in a 16/8 h photoperiod during 15 days.

2.2. In Vitro Culture Induction

In vitro tissue cultures were induced from the germinated plantlets on MS media [9] supplemented with auxins for root induction and cytokinins for callus induction. For root induction in marigold and *Heliopsis*, different concentrations of auxins (IBA: 0, 100, 250, 500, 1000, 2000 mg/mL) were tested. For callus induction, combinations of auxins (NAA: 0, 500, 1000 mg/mL) and cytokinins (BA: 0, 500, 1000 mg/mL) were tested in marigold.

2.3. Total RNA Extraction

Tissue from roots and calli were collected, frozen in liquid nitrogen and powdered. RNA was extracted with trizol (Invitrogen) as described by the manufacturer. Then, 100 mg of powdered tissue was used, the RNA extracts were precipitated with lithium chloride and resuspended in 50 uL of RNase free sterile water. The RNA concentration was calculated and integrity analyzed by 1% agarose gel electrophoresis.

The miRNA identification on the plant tissue was determined by RT-PCR stem loop as described by [10], the products were analyzed in 4% agarose. The selected tissues include the following: leaves, stem and roots for *Heliopsis*, and flower, buds, leaves, callus and roots in marigold.

3. Results and Discussion

3.1. In Vitro Culture

The first attempt to establish the in vitro root culture in marigold included the following different tissues: leaves and stems. In leaves, no root formation was shown, in contrast to stem tissue, where adventitious root was developed. In previous assays, a better response to IBA was also found, over 2,4-D. Then, with these results, a root induction kinetical with auxins was assayed, using IBA at different concentrations (0, 100, 250, 500, and 1000 µg/mL). In Table 1, the result for the kinetical assays for root induction is shown. As observed, the response starts from 100 µg/mL, and the highest value is at 1000 µg/mL, with 10.58 at the root formation coefficient (rfc), then decay. The structure is shown as a principal root with an abundant development of secondary roots.

In the case of callus induction, the best response was obtained with 1 mg of ANA and 0.5 mg of BA; a firm callus was recovered, and the multiplication in ANA was possible. The total RNA was isolated from these tissues for miRNA identification.

Table 1. In vitro culture for marigold roots induction.

	0	100	250	500	1000	200 µg/mL
Media	0	10	88	127.5	212	212
Total	0	54	17	21	23	23
RFE	0	11	34	42	46	46
% RFE	0	22	5.78	8.82	10.58	10.58
RFC	0	2.42	88	127.5	212	212

Seeds were germinated on MS media, hypocotyls were cut in fragments of 0.5 cm and incubated on Petri dishes with different concentrations of MS. The Plates were incubated for 15 days when data were registered as number of explants with roots and number of roots in each explant.

For *Heliopsis*, the assay was developed using the same concentrations of IBA (0, 100, 250, 500, and 1000 µg/mL). An increase from the 250 µg/mL concentration was observed until 1000 µg/mL, where the maximal development was reached, with 5.8 RFC (not shown). The root structure was different from marigold, as it shows just a single root, without secondary roots (not shown).

3.2. miRNAs Expression Analysis

The results for the amplification for miRNA in *Heliopsis longipes* are shown in Table 2; five miRNAs showed specific expression on the roots (miR156, miR164) and stems (miR159, miR168, miR171), which suggests a specific regulation in the organ, and possibly in the metabolism, as indicated in Table 2. As shown, miRNA in chilcuague seems to be associated with the synthesis of important secondary metabolites, including others such as taxol. It will be interesting to analyze the metabolite production and its association with miRNA expression.

Table 2. Induced miRNAs in *Heliopsis longipes*.

miRNA	Organ Expression	Target	Metabolic Pathway
miR156	Root	Dihydroflavonol 4-reductase SQUAMOSA (SPL), AP1 Transcription factors	Anthocyanins, synthesis (flavons, flavonols), terpenoids (carotenoids)
miR159	Stem	GAMYB (R2R3 MYB) Transcription factors	Giberellic acid transduction Flavonoids synthesis
miR164	Root	Taxano 13 α -hydroxylasa, Taxano 2 α -O benzoyltransferasa	Taxol synthesis
miR168	Stem	Acetil-CoA acetyltransferase	Terpenoids synthesis
miR171	Stem	Protochlorophyllide oxidoreductasa, Taxano 13 α -hydroxylasa y, Taxano 2 α -O benzoyltransferasa	Giberelins, carotenoides, flavonoides and taxol synthesis

In marigold, miRNA analysis showed differential expression in callus, and the root tissue culture. It was showed specific expression of miRNAs in the root or callus (Table 3). Three miRNAs were found with expression on callus culture (miR159, miR165, miR167), and two with expression in root culture (miR164, miR168).

Table 3. miRNAs expression in marigold.

miRNA	In Vitro Culture
miRNA159	Callus
miRNA164	Roots
miRNA165	Callus
miRNA167	Callus
miRNA168	Roots

4. Conclusions

In vitro culture systems were developed for marigold (callus and root culture) and *Heliopsis longipes* (root culture). The miRNAs associated with secondary metabolite production were determined, as well as the targets and possible metabolic pathways. With this information, it will be possible to establish a system for in vitro manipulation and production of metabolites of interest.

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