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

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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 in the animal and human health, has been make them a target for biotechnological improvement. At the región Bajio in Mexico, have been identified several plants associated with ecological, medical and industrial potential, but also asocciated to the traditions. The work in this Project includes the development of systems for the culture for production of secondary metabolites (in vitro root tissue culture) and the miRNA expresión analysis in order to find the molecules asocciated to metabolites production. In this study we include two plants: marigold (*Tagetes erecta*), which genes associated to lutein production had been identified in flower development, systems for cell culture and plant transfromation has been developed, but no systems for in vitro root culture. To now there are not studies related to miRNA expresión and association to these molecules to secondary metabolites. In *Heliopsis longipes*, several metodologies 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 cinetical 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 differentially expressed and associated to secondary metabolites production (miR146, miR164, miR168, miR171). The reordering of miRNAs synthesis and the targets was analysed and is associated to 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

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

Marigold (*Tagetes erecta*), has been cultivated since the Antique, mainly as ornamental. The plant is used in religious ceremonias, Also their uses in the pharmaceutical área is associated such as antiparasitic, antispasmodic and disease-fighting (1). In marigold, the genes associated to lutein production has been isolated and characterized (2), also the tissue culture and genetic transformation

was (3). No data associated to secondary metabolites expression and development are reported yet, even no reports on root culture exist.

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

miRNAs are a class of non-codificant small RNAs tha regulate the gene expression in eucariotes. They are involved in different plant development processes, and different disease response mechanism and stress (6). miRNAS plays an essential in the postranscriptional 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, is necessary to analize their expression and their targets, which have a negative correlationship. The contundent evidence for miRNA function is the expression in transformed plants, were is posible to evaluate their effect on specific processes. Recently miRNAs study has been focus on secondary metabolism and it was posible to correlate the function of miRNAs to secondary metabolites biosynthesis, the miRNAs induced in root and flowers of marigols 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 merigold and Heliopsis were desinfested with absolute etanol, 20% sodium hipochlorite washed with sterlie wáter and exposed to an antifungal compound (PPM), during 12 h, then washed and transfer 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), suplemented with auxins for root induction and citokinins fos calli induction. For root induction in marigold and Heliopsis, different concentrations of auxins (IBA: 0, 100, 250, 500, 1000, 2000 mg/mL) were tested. For calli induction combinations of auxins (NAA: 0, 500, 1000 mg/mL and: citokinins (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 poder. RNA was extracted with trizol (In vitrogen) as described by the manufacturer. 100 mg of poder tissue were used, the RNA extracts were precipitated with litium chloride and resuspended in 50 ul of RNAses free sterile water. The RNA concentration was calculated and integrity analyzed by 1% agarose gel electrophoresis.

2.4. miRNA RT-PCR Setm Loop Expression Analysis

The miRNA identification on the plant tissue was determined by RT-PCR stem loop as described by (10), the products wer analyzed in 4% agarose. The selected tissues include: leaves, stem and roots for Heliopsis and Flower, buds, leaves, calli and roots in marigold.

3. Results and Discussion

3.1. In Vitro Culture

The first attempt to stablish the in vitro root culture in marigold included the differnt tissues: leaves and stems. In leaves no root formation was shown, in contrast to stem tissue were adventicial root was developed. In a previos assays, also a better response to IBA was found over 2,4-D; Then With these results, an induction cinetical with auxins was assayed.using IBA at diferent concentration (0,100, 250, 500 and 1000 ug/mL). In Table 1 is shown the result for the cinetical assays for root induction, As seen, from 100 ug/mL starts the response and the highest value is at 1000 ug/mL with 10.58 at root formation coeficient (rfc), then decay. Th estructure is shown as a principal root with an abundant development of secondary roots.

In the case of calli induction, the best response was obtained with 1mg of ANA and 0.5 mg of BA, a firm calli was recovered, the multiplication in ANA was posible. From these tissues was isolate the total RNA for miRNA identification.

Table 1. In vitro culture for marigold roots.

	0	100	250	500	1000	200 ug/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, hipocotils were cut in fragments of 0.5 cm and incubate in Petri dishes (5) with differnt concentrations of MS. Plates incubate for 15 days when data was registrated 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, 1000 ug/mL). An increase from the 250 ug/mL concentration was observed until 1000 ug/mL were reach the maximal development with 5.8 RFC (not shown). The root structure was different from marigold, it shows just a single root, with out secondary roots (not shown).

3.2. miRNAs Expression Analysis

The results for amplification for miRNa in *Heliopsis longipes* are shown in Table 2, five miRNA shown specific expression on roots (miR156, miR164) and stems (miR159, miR168, miR171), it suggests a specific regulation in the organ and posible in the metabolism as indicate in the Table 2. As shown, miRNA in chilcuague seems to be associated to the synthesis of important secondary metabolites, including others such as taxol, it will be interesting to analyze the metabolite production and its asociation to 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	Anthocianins, synthesis (flavons, flavonols), terpenoids (carotenoids)
miR159	Stem	*GAMYB* (R2R3 MYB) Transcription factors	Giberellic acid transduction Flavonoids synthesis

miR164	Root	Taxano 13α-hydroxylasa, Taxano 2α-O benzoiltransferasa	Taxol synthesis
miR168	Stem	Acetil-CoA acetyltransferase	Terpenoids synthesis
miR171	Stem	Protochlorophyllide oxidoreductasa, Taxano 13α-hydroxylasa y, Taxano 2α-O benzoyltransferasa	Giberelins, carotenoides, flavonoides and taxol synthesis

In marigold, miRNA analysis in marigold shown differental expression in calli and root tissue culture shown specific exprssion of miRNAs in root or calli (Table 3). Three miRNAs were found expression on calli (miR159, miR165, miR167 culture, two in root (miR164, miR168).

Table 3. miRNAs expression in marigold.

miRNA	In Vitro Culture
miRNA159	Callo
miRNA164	Raiz
miRNA165	Callo
miRNA167	Callo
miRNA168	Raiz

4. Conclusions

In vitro culture systems were developed for marigold (callus and root culture) and Heliopsis longipes (root culture). miRNAs associated to secondary metabolite production was determined, also the targets and posible metabolic pathway. With these information will be possible to stablish a system for in vitro mnipaltion and protuction of metabolites of interest.

Author Contributions: A.C.-H.A. conceived the Project and designed the experiments. S.J.-M., D.C.-P. and A.A.-P. performed the experiments, A.C.-H.A. and A.A.-P. analyzed the data. A.C.-H.A. wrote the manuscript with inputs from all co-authors. All authors have read and agreed to the published version of the manuscript.

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References

1. Cruz-Ramírez, L.A.; Valdez-Morales, M.; Chacón-López, M.A.; Rosas-Cárdenas, F.F.; Cruz-Hernández, A. Mexican crops of Agroalimentary importance. In *Advances in Agricultural and Food Biotechnology*; Guevara González, R., Pacheco, T., Eds.; Research Signpost: Kerala, India, 2006; ISBN: 81-7736-269-0.
2. Del Villar Martínez, A.A.; García-Saucedo, P.A.; Carabez-Trejo, A.; Cruz-Hernández, A.; Paredes-López, O. Carotenogenic gene expression and ultrastructural changes during marigold flowering. *J. Plant Physiol.* **2005**, *162*, 1046–1056, doi:10.1016/j.jplph.2004.12.004.
3. Vanegas-Espinoza, P.E.; Valdez-Morales, M.; Valverde, M.E.; Cruz-Hernández, A.; Paredes-López, O. Particle bombardment, a method to gene transfer in marigold. *Plant Cell Tiss Org. Cult.* **2006**, *84*, 359–363, DOI 10.1007/s11240-005-9030-x.
4. Molina-Torres, J.; Salgado-Garciglia, R.; Ramírez-Chávez, E.; del Río, R.E. Purely Olefinic Alkamides in *Heliopsis longipes* and Acmella (Spilanthes) oppositifolia. *Biochem. Syst. Ecol.* **1996**, *24*, 43–47.
5. Ruiz, C.; Rojas-Molina, A.; Luna-Vázquez, F.J.; Rivero-Cruz, F.; García-Gasca, T.; Ibarra-Alvarado, C. Affinin (Spilanthol), Isolated from *Heliopsis longipes*, Induces Vasodilation via Activation of Gasotransmitters and Prostacyclin Signaling Pathways. *Int. J. Mol. Sci.* **2017**, *18*, 218, doi:10.3390/ijms18010218.
6. Wei, S.; Gruber, M.Y.; Yu, B.; Gao, M.-J.; Khachatourians, G.G.; Hegedus, D.D.; Hannoufa, A. Arabidopsis mutant sk156 reveals complex regulation of SPL15 in a miR156-controlled gene network. *BMC Plant Biol.* **2012**, *12*, 169, doi:10.1186/1471-2229-12-169.

7. Cedillo-Jiménez, C.A.; Hernández–Salazar, M.; Escobar-Feregrino, T.; Caballero-Pérez, J.; Arteaga-Vázquez, M.; Cruz-Ramírez, A.; Torres-Pacheco, I.; Guevara-González, R.; Cruz-Hernández, A. MicroRNAs Sequencing for Understanding the Genetic Regulation of Plant Genomes. In *Plant Genomes*; Intech Publishing: 2016; ISBN 978-953-51-4622-3.
8. Vargas-Hernández, M.; Vázquez-Marrufo, G.; Aguilar-Ruiz, C.; González-Márquez, M.A.; Rocha, O.; Cerna-Pantoja, D.; Cruz-Hernández, A. MicroRNAs Associated with Secondary Metabolites Production. In *Plant Physiological Aspects of Phenolic Compounds*; Intechopen: 2019; ISBN 978-1-78984-034-6.
9. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 472–497.
10. Li, H.; Zhang, Z.; Huang, F.; Chang, L.; Ma, Y. MicroRNA expression profiles in conventional and micropropagated strawberry (*Fragaria* × *ananassa* Duch.) plants. *Plant Cell Rep.* **2009**, *28*, 891–902, doi:10.1007/s00299-009-0693-3.

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