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Callus culture development of two varieties of *Tagetes erecta* and carotenoid production

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ABSTRACT

Background: The properties of natural pigments, such as antioxidants, functional, medical, and nutraceutical, have demonstrated the advantages of these natural compounds over synthetic ones. Some products are accepted only when they are pigmented with natural, food-quality colorants: for example poultry products (manly marigold flower extracts). Carotenoids such as β-carotene, β-criptoxanthin and lutein are very attractive as natural food colorants due to their antioxidant and pro-vitamin activities which provide additional value to the target products. Marigold (*Tagetes erecta*) is an Asteraceous ornamental plant native to Mexico, and it is also important as a carotenoid source for industrial and medicinal purposes but nowadays its production is destined mainly for ornamental purposes.
Results: Friable callus of *T. erecta* yellow flower (YF) and white flower (WF) varieties was induced from leaf explants on Murashige and Skoog (MS) medium supplemented with 9.0 μM 4-dichlorophenoxyacetic acid (2,4-D) and 8.8 μM benzyladenine (BA). Calluses developed from both varieties were different in pigmentation. Extract characterization from callus cultures was carried out by high-performance liquid chromatography (HPLC). This analytical process detected several carotenoids; the main pigments in extracts from YF callus were lutein and zeaxanthin, whereas in the extracts of the WF callus the main pigments were lutein, zeaxanthin, β-cryptoxanthin and β-carotene. Callus cultures of *T. erecta* accumulated pigments even after several rounds of subculture.
Conclusions: WF callus appeared to be a suitable candidate as a source of different carotenoids, and tested varieties could represent an alternative for further studies about in vitro pigment production.
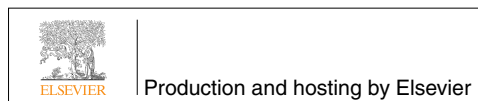
1. Introduction

Natural pigments are involved in essential processes for the plant life such as photosynthesis, plant protection and reproduction among others [1,2]. Carotenoids, a large group of fat-soluble natural pigments of higher plants and other organisms, are responsible for the yellow, orange, and red colors in plants, animals, bacteria, and fungi [3]. The carotenoid biosynthesis pathway has been widely studied and it is important to continue analyzing it in different plant sources [4]. These compounds are the second most abundant pigments in nature and are involved in an important set of reactions in plant reproduction, through their role in attracting pollinators and seed dispersers. It is well recognized that the main function of these pigments in green tissues is protecting photosynthetic apparatus [5]. The nature annual production of carotenoids is about 100 million tons [6] and they are of pharmaceutical interest for uses not only as natural food colorants, but also by playing an important role in human nutrition and health [2]. Furthermore, the intake of carotenoid-rich products has been associated to lower incidence of certain disorders like cancer and cardiovascular illnesses [7,8]. Lutein and zeaxanthin are macular pigments that might play an important role by reducing development and progression of age-related macular degeneration [9,10]. Carotenoid analysis involves extraction, saponification, separation, and characterization, for both identification and quantification. Best results have been obtained by high-performance liquid chromatography (HPLC), which is the most efficient method for the qualitative and quantitative analyses of carotenoids. The efficiency of this technique has been improved by diode array detector (DAD) allowing detection at several wavelengths and simultaneous putative identification by UV spectral analyses [4,11].

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A number of research works on this subject have been reported over the last few years to deal with the optimization of carotenoid production methodologies [12]. Thus, obtaining pigments through plant cell tissue culture, microbial fermentations, and genetic manipulation has been investigated. Current trends in industrial production have focused on introducing environmentally safer biotechnological processes. Bacteria (*Flavobacterium multivorum*, *Brevibacterium linens*), fungi (*Phaffia rhodozyma*, *Blakeslea trispora*, *Phycomyces blakesleanus*) and microalgae (*Haematococcus pluvialis*, *Dunaliella salina*) have been considered for carotenoid production [13]. Carotenoid production from plants frequently involves extraction from different plant tissues, being often tedious and costly due to low yields, and the target compounds may be only seasonally available [14]. Plant cell culture methodologies have the potential to overcome many of these problems. A number of publications to date have succeeded in producing a wide range of valuable secondary metabolites from unorganized callus or suspension cultures such as ginseng (*Panax ginseng*) and taxol (*Taxus baccata*) [15].

Large scale plant tissue culture has been demonstrated as being an alternative approach to traditional crops as it offers controlled supply of metabolites, independent of plant availability [16]. Due to these advances, research in the area of tissue culture technology for production of plant chemicals has bloomed beyond expectations, producing useful compounds under controlled conditions independent of climatic changes or soil status, free of microbes and insects [17,18]. In this sense, plant tissue culture has been used for production of several carotenoids, in different plants to simplify breeding procedures and address agronomic and environmental problems facing crop production [16,19]. Gao et al. [20] and Xu et al. [21] reported carotenoid production from the callus of different citrus varieties stating that this callus may help to understand the pigmenting mechanism in the future. Furthermore, Vanegas-Espinoza et al. [22] observed that carotenoids in *T. erecta* cells, mainly lutein, were accumulated in plastoglobular substructures within the chromoplast, showing by HPLC analysis, the presence of carotenoids in callus extracts. These studies reveal support that a continuous system of cell proliferation as callus culture is a useful tool for the study of carotenoids since they have acquired importance because of their biological functions.

There are few biotechnological researches referring to the cellular pigment content [22]. The wide range of uses of *T. erecta* underlines the importance of establishing a reliable plant tissue culture system for *in vitro* carotenoid production. In this work, we describe the establishment of a cell culture from two contrasting pigment varieties (yellow and white flowers) of *T. erecta*, in order to qualitatively compare pigment presence and accumulation for their further utilization in breeding strategies to modify carotenoids biosynthetic pathway and their cellular storage.

2. Materials and methods

2.1. Plant material

Plantlets were obtained by seed germination of two marigold (*T. erecta*) varieties: yellow flowers (YF) donated by Dr. Miguel Ángel Serrato-Cruz from Universidad Autónoma Chapingo, Texcoco, Estado de México, México and white flowers (WF), from Plántulas de Tetela S. de R.L. de C.V., Cuernavaca, Morelos, México. Disinfestation of three lots of 20 seeds from each variety was performed as follows: ethanol (100%) for 1 min, ethanol (70%) for 5 min, sodium hypochlorite (2%) for 15 min and sodium hypochlorite (1%) for 15 min. Seeds were rinsed for 3 min in sterile distilled water between each treatment [19]. Disinfested seeds were germinated on Murashige and Skoog (MS) medium [23] supplemented with sucrose (30 mg/l) and 3 g/l Phytagel (Sigma-Aldrich, USA). pH was adjusted to 5.8, and seed germination was conducted in baby-food flasks (8 cm h × 6.2 cm d, 190 ml) incubated in a growth chamber at $25 \pm 2°C$ under fluorescent light (50 μmol/m^2 s^{-1}) and 16/8 h light/dark photoperiod cycle.

2.2. Germination test

The germination index and germination velocity were calculated according to Enríquez-Peña et al. [24], using the following equations:

$$IG = \frac{\sum (n_i t_i)}{N} \qquad \text{[Equation 1]}$$

where:

IG   germination index
n_i number of germinate seeds in the day *i*
t_i d after initiating culture
N   total number of planted seeds.

$$M = \sum \frac{(n_i)}{t} \qquad \text{[Equation 2]}$$

where:

M   germination velocity
t   time of germination after initiating culture until germination of the last seed.

2.3. Callus induction and maintenance

Three week-old plantlets were used as a source of explants for callus induction. Young leaves were cut in portions of about 0.25 cm^2 and the adaxial face was placed on the MS medium described above, supplemented with all combinations of 2,4-dichlorophenoxyacetic acid (2,4-D; 4.5, 9.0, 13.5 μM) and benzyl adenine (BA; 2.2, 4.4, 8.8 μM), according to Vanegas-Espinoza et al. [22]. Cultures were incubated in the dark at $25 \pm 2°C$ for three weeks. Callus induction percentages were determined after three weeks of culture. Obtained calluses were continuously subcultured every 15 d to fresh MS medium, supplemented with 2,4-D/BA concentrations defined in the experiment above, to reduce the browning of callus.

2.4. Callus growth evaluation

In order to study kinetic growth of marigold callus, a culture was started with 0.2 g of callus. Cultures were kept in the dark at $25 \pm 2°C$ and evaluated after 18 d. Callus samples were dried to constant weight at 40°C for 24 h, and growth was measured in relation to dry weight. Doubling time growth was calculated with a graphic method using the equations:

$$Td = \frac{\ln 2}{m} \qquad \text{[Equation 3]}$$

where:

Td   doubling time growth
ln2   natural logarithm
m   slope of the line.

Cell growth data in the form of natural logarithm was plotted versus time. This yields a straight line over the exponential phase of growth. The slope of the linear part of the curve corresponds to μ and is given in 1/unit of time [25].

2.5. Pigments analysis

Carotenoid extraction was performed according to Vanegas-Espinoza et al. [20] with some modifications, 0.2 g of lyophilized callus

was extracted with 3 ml of hexane:absolute ethanol:acetone:toluene (10:6:7:7 v/v/v/v), and 40% methanolic KOH solution for 4 h in darkness and constant agitation. The chromatographic analyses were performed on an Agillent system comprising a quaternary pump, a diode array detector (DAD), a column temperature control module, and an autosampler (Agilent 1100, Palo Alto, CA). The separations were achieved on a C30 column (5 µl, 250 b × 4.6 mm) (YMC, Wilmington, NC) kept at 17°C. Methanol (MeOH), tert-butyl methyl ether (TBME) (both containing 0.1% of butylated hydroxytoluene and 0.05% of triethylamine) and water were used in the mobile phase according to the following linear gradient: 90% MeOH + 5% TBME + 5% water; 12 min: 95% MeOH + 5% TBME; 25 min: 89% MeOH + 11% TBME; 40 min: 75% MeOH + 25% TBME; 60 min: 50% MeOH + 50% TBME; and 62 min: 90% MeOH + 5% TBME + 5% water. The monitoring of the peaks was carried out at 430, 450 and 486 nm and the flow and sample volumes drawn were set at 1 ml/min and 20 µl, respectively. Criteria for pigment identification included retention time, UV spectra at 450 nm using diode array detector compared to carotenoid standards (lutein, β-carotene, astaxanthin and zeaxanthin standards; Sigma-Aldrich, USA) and absorbance maxima from spectra as described by Meléndez-Martínez et al. [26].

2.6. Statistical analysis

Factorial analysis was carried out in order to analyze the effect of treatments and their interactions. Fisher's least significant difference ($p = {<}0.05$) was applied to show statistical significance on differences among the means.

3. Results

3.1. Germination and callus induction

Seeds of both *T. erecta* varieties germinated after 5 d of culture on free MS medium. Plantlet elongation and cotyledonal leaf development were observed after 5 d of culture and foliage leaves appeared after 10 d (Fig. 1). Germination of YF variety was 90%, IG = 4.5 d and M = 12.5 seeds/d, while WF presented 83% germination, IG = 4.4 d and M = 3.3 seeds/day. Leaves from these seedlings were used as initial explant for callus induction on MS medium with combinations of 2,4-D and BA.

During the first week of culture both explant varieties remained green. After that, explants lost pigmentation because of dedifferentiation process; on the second week callus formation was evident, and on the third week they were completely undifferentiated. Callus induction was different for both varieties at the 15th day and was classified as either compact or friable. Percentage of callus induction is shown in Table 1; calluses from YF explants were friable and bright yellow on MS 2,4-D (9.0 µM) and BA (8.8 µM) and those from WF explants were friable and pale yellow under the same conditions (Fig. 2a and b). Nevertheless other treatments produced higher callus induction, but

Table 1
Effect of 2,4-D and BA on callus induction from leaf explant of *Tagetes erecta* varieties of yellow flowers (YF) and white flowers (WF) after 15 d culture.

Plant growth regulator (µM)		YF callus induction	WF callus induction
2,4-D	BA	(%)	(%)
4.5	2.2	10.0[c]	30.0[d]
	4.4	19.5[b]	66.6[c]
	8.8	10.0[c]	70.0[bc]
9.0	2.2	10.0[c]	36.6[d]
	4.4	8.2[c]	83.3[a]
	8.8	32.0[a]	73.3[b]
13.5	2.2	4.9[d]	76.0[b]
	4.4	8.0[c]	86.6[a]
	8.8	6.0[cd]	76.6[b]

Values with different upper case letters are statistically different within columns ($P < 0.05$).

they were compact and brownish, with nodular structures which could be the subject of another study, which would examine the possibility of inducing somatic embryos (Fig. 2c and d).

Callus induction was observed on leaf explant after 10 d of culture on MS 2,4-D (9.0 µM) and BA (8.8 µM) (Fig. 3). After four subcultures to fresh medium calluses maintained their friability and showed different pigmentations (Fig. 4).

3.2. Callus growth and production of carotenoids

The growth curve of both cell cultures is illustrated in Fig. 5. The culture growth was developed in two phases: (adaptation and growth) during 18 d of culture on MS medium added with 2,4-D (9.0 µM) and BA (8.8 µM). The adaptation phase of YF callus was observed from 0 to about 11 d of subculture without an important increase in dry weight and, the growth phase was observed from d 11 up to 18 d (Fig. 5a). The doubling time of YF callus culture was 3.3 d, and the growth rate was 0.33 d⁻¹. Meanwhile the adaptation phase of the WF callus culture was observed at about 7 d and there was an increased growth phase from that moment up to 17 d (Fig. 5b). The doubling time of WF callus culture was 3.3 d, and the growth rate was 0.29 d⁻¹. Based on the features of culture growth of both varieties, calluses were continuously subcultured every 15 d on fresh medium maintaining their friability and reducing the browning of callus.

In order to study the carotenoid contents within calluses of YF and WF varieties, carotenoid content of both calluses was HPLC analyzed (Fig. 6). HPLC profile of the YF variety callus showed two main peaks (Fig. 6a). Lutein was eluted at 23.4 min, and zeaxanthin at 26.5 min. Pigment profiles corresponded to absorbance spectra (Fig. 6c). HPLC profile of the WF callus showed a main peak at 23.6 min corresponding to lutein and some minor peaks at 26.1, 37.6 and 47.2 min, which match with retention times and absorption spectra corresponding to zeaxanthin, β-cryptoxanthin and β-carotene, respectively (Fig. 6b). Lutein was the main pigment found in both extract varieties.
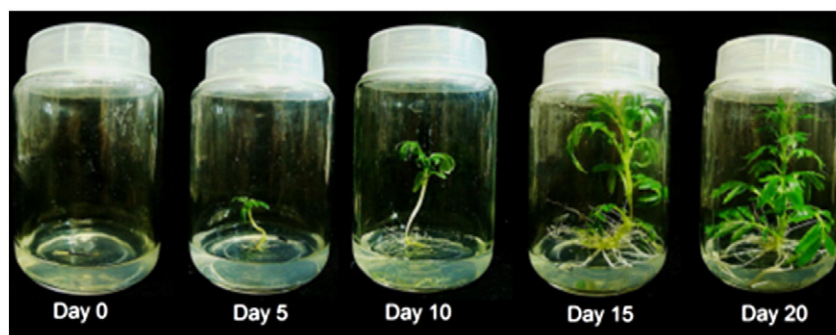

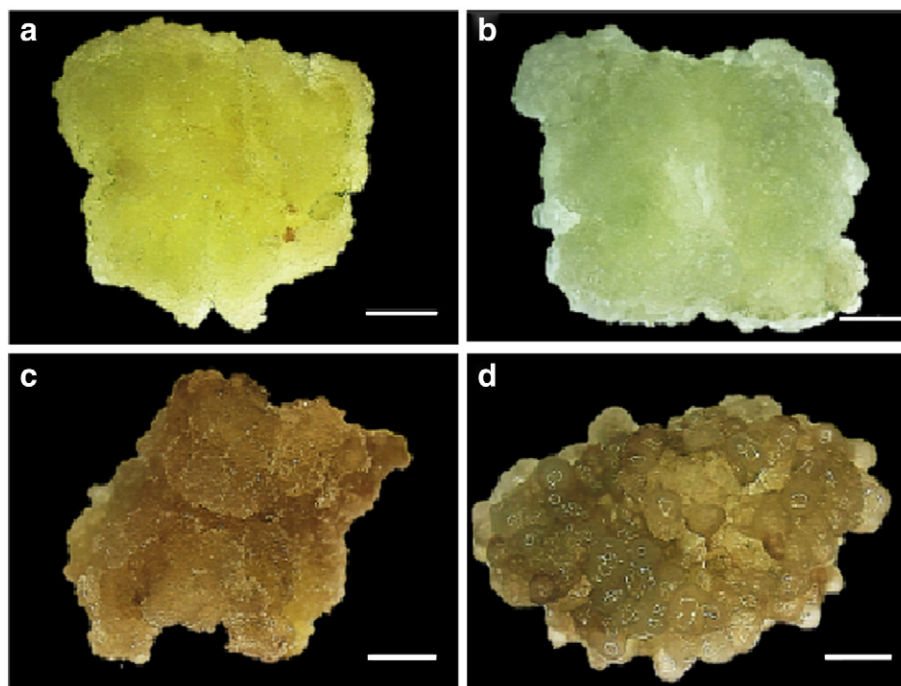**Fig. 1.** Process of germination and seedling development of *T. erecta* during 20 d.

Fig. 2. Appearance of callus induced from *T. erecta* leaves after 15 d of culture. In detail: (a) and (b) Friable callus from YF and WF explants, respectively, on MS 2,4-D (9.0 μM)/BA (8.8 μM); (c) compact callus from YF on MS 2,4-D (4.5 μM)/BA (8.8 μM); and (d) compact callus with nodular structures from WF explants on MS 2,4-D (4.5 μM)/BA (8.8 μM). Bar represents 1.0 mm.

4. Discussion

In this study, two varieties of *T. erecta* with different inflorescence pigmentations have been *in vitro* screened for their possibility to undergo carotenoid production from callus culture, and contribute to the understanding of the regulation of the carotenoid biosynthesis pathway in this kind of cell. Leaf explants have proved to be an adequate tissue to establish cell cultures, because of the presence of a meristematic tissue in young leaves, allowing continuous mitotic division, which is increased when tissues are exposed to auxin and cytokinin in the culture medium [27]. 2,4-D (9.0 μM) and BA (8.8 μM) were found to be the suitable combination for friable callus induction; this was demonstrated in terms of a high induction percentage. Although there were media with higher callus induction, friable callus was induced on mentioned concentrations; this variation in callus induction/differentiation on different concentrations of growth regulators depends on the morphogenetic response which varies according to spatial and temporal cell distributions, and on physiological and developmental stages [28]. Endogenous concentrations of growth hormones and tissue response is genetically regulated [29], and the use of young leaves as a source of explant was previously reported in marigold (*T. erecta*) by Misra and Datta [30]. Nodular callus was observed with all the concentrations of 2,4-D and BA in this study, and these structures have been reported as early stages of somatic embryogenesis [31]. Kandasamy et al. [32] suggested that medium hormonal composition plays an important role in determining the type of cells of the explant which would undergo division, phytohormones are evidently involved in the switch-over from the mitotic cell cycle to the endomitotic cell cycle and vice versa, auxin generally stimulates DNA synthesis, cell division, cell expansion, cell differentiation, and organ initiation while cytokinin acts as a trigger for mitosis and callus proliferation [33], and auxins and cytokinins like 2,4-D and BA are also known to induce the rapid synthesis of specific mRNAs and proteins suggesting that they are necessary to regulate these growth processes. Exogenous application of hormones initiates a variety of biochemical events, that culminate in processes directed to the cytoskeleton by the activation of the family of transcription factor genes, called "auxin response factors" and "cytokinin response factors" [34,35]. Moreover, the concentration of exogenous
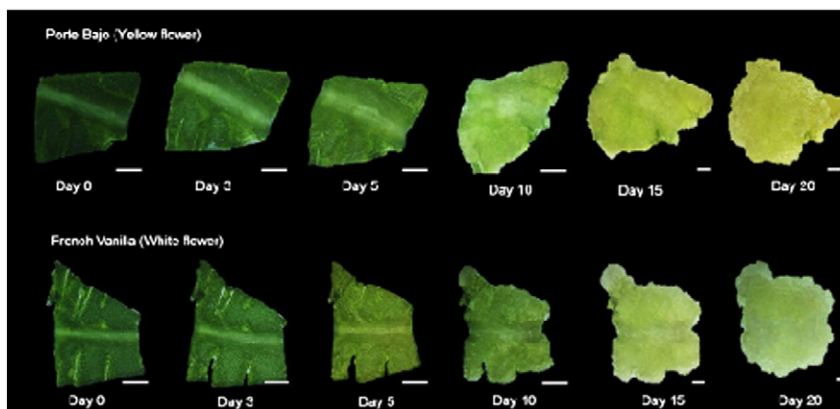

Fig. 3. *T. erecta* callus induction process from YF (yellow flowers) and WF (white flowers), on MS added with 9.0 μM 2,4-D and 8.8 μM of BA. In detail: Bar represents 1.0 mm.
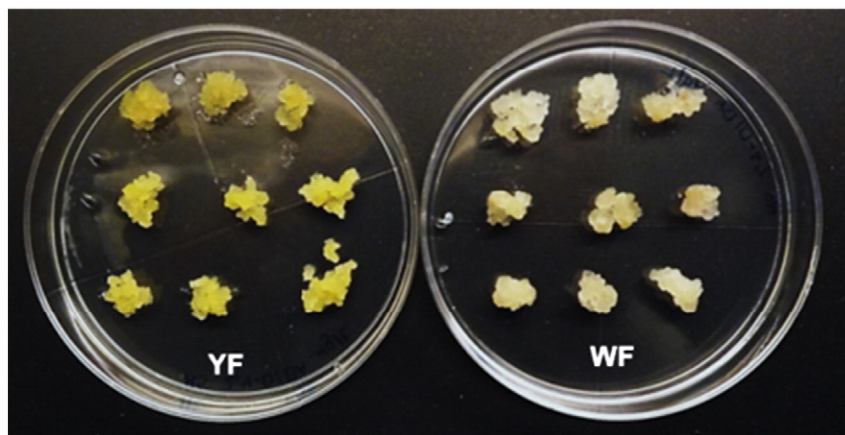
Fig. 4. Callus obtained from leaf explant of *T. erecta* variety on MS supplemented with 2,4-D (9.0 μM) and BA (8.8 μM). YF (yellow flowers) and WF (white flowers).

and endogenous phytohormones can inhibit the induction of the undifferentiation process by competition of specific receptor; these competitions interfere with the metabolism of DNA and RNA [36]. Tested 2,4-D/BA combinations showed different responses like the degree of compaction of callus, and unfriable/friable callus, and these effect were influenced by auxin and cytokinin rate during cell division, cell elongation and cell differentiation, although exactly how they are involved in each process is not completely understood. However, *Arabidopsis thaliana* callus induced by hormones shows enhanced actin gene expression containing several putative hormone response DNA sequence elements; i.e. *act7* gene constitutes the predominant actin gene in tissue culture cells, and has an essential role to play during hormone induced callus formation [32]. The different effects observed on the explants of both *T. erecta* varieties tested was probably influenced by expression of actin

genes and transcription factors that respond to several external stimulation including concentration of 2,4-D/BA tested.

Though 2,4-D (4.5 μM) and BA (8.8 μM) stimulated the formation of two kinds of calluses showing different morphologies; one of them with nodular structures could be related with embryogenic callus formation [37]. Michelangeli de Clavijo et al. [38] reported in *Bixa orellana* L. cultures, proembryos (nodular structures), spherical structures, with cells of variable shape and size, originating from callus surface, after the initial stages globular and heart-shaped somatic embryos was observed. Embryogenic callus induction and somatic embryo development from cotyledonary explants of *T. erecta* on 2,4-D (4.5 μM)/kinetin (0.93 μM) medium were reported previously by Bespalhok and Hattori [39].

The main carotenoids in the extracts from the YF callus were lutein and zeaxanthin, whereas those in the extracts of the WF callus were lutein, zeaxanthin, β-cryptoxanthin and β-carotene. Lutein was the main pigment in both calluses (YF and WF). It is important to point that lutein accumulation occurs not only in specialized tissue, it has been reported that on undifferentiated tissue (callus), chromoplasts are the organelles where carotenoid synthesis and storage occurs [22]. Interestingly, lutein content diminished in callus from WF; on marigold ligules, a similar pattern was observed before with a yellow variety that accumulates high concentrations of lutein, while the white flower almost abolished lutein accumulation [14]. Natural pigment production by cell culture has been previously reported [1,40]. Callus culture of *T. erecta* accumulated pigment even after several rounds of subculture. WF callus accumulated also β-carotene, β-cryptoxanthin and zeaxanthin, and it could be an alternative source for pigment production; since they are better pigmenting agents for egg yolk and broiler skin than lutein, [41]. Several plants (*Capsicum annuum*, *Lycium barbarum*, *Hippophae rhamnoides*, *Physalis alkekengi*, *Diospyros kaki* and *Cucurbita pepo*) have been studied because of their high carotenoid content and have been proposed as source of these compounds for industry [42,43], therefore *T. recta* callus as an important source of lutein and other carotenoids such as zeaxanthin would be an option for food or pharmaceutical industry.

Selection of highly producing systems of carotenoids, by means of *T. erecta* cell culture establishment, could be the basis for their use to produce other biochemical compounds using alternative techniques such as biosynthetic pathway induction and/or genetic modification which have proven to be efficient to increase the accumulation of secondary metabolites.

Fig. 5. Callus growth of *Tagetes erecta* varieties. YF (a) and WF (b) on MS medium supplemented with 2,4-D (9.0 μM) with BA (8.8 μM). Time courses of biomass accumulation of YF and WF callus culture at 18 d. Each dot and bar shows the average ± SD of three independent samples.

5. Concluding remarks

This work reports the qualitative comparison of carotenoid production in callus from two *T. erecta* varieties with contrasting pigmentation (YF and WF), and it was shown that both varieties had
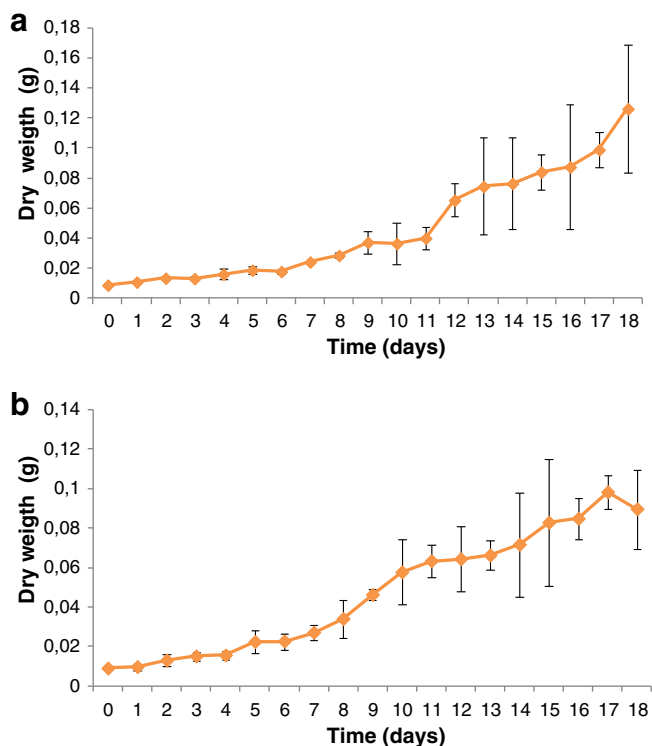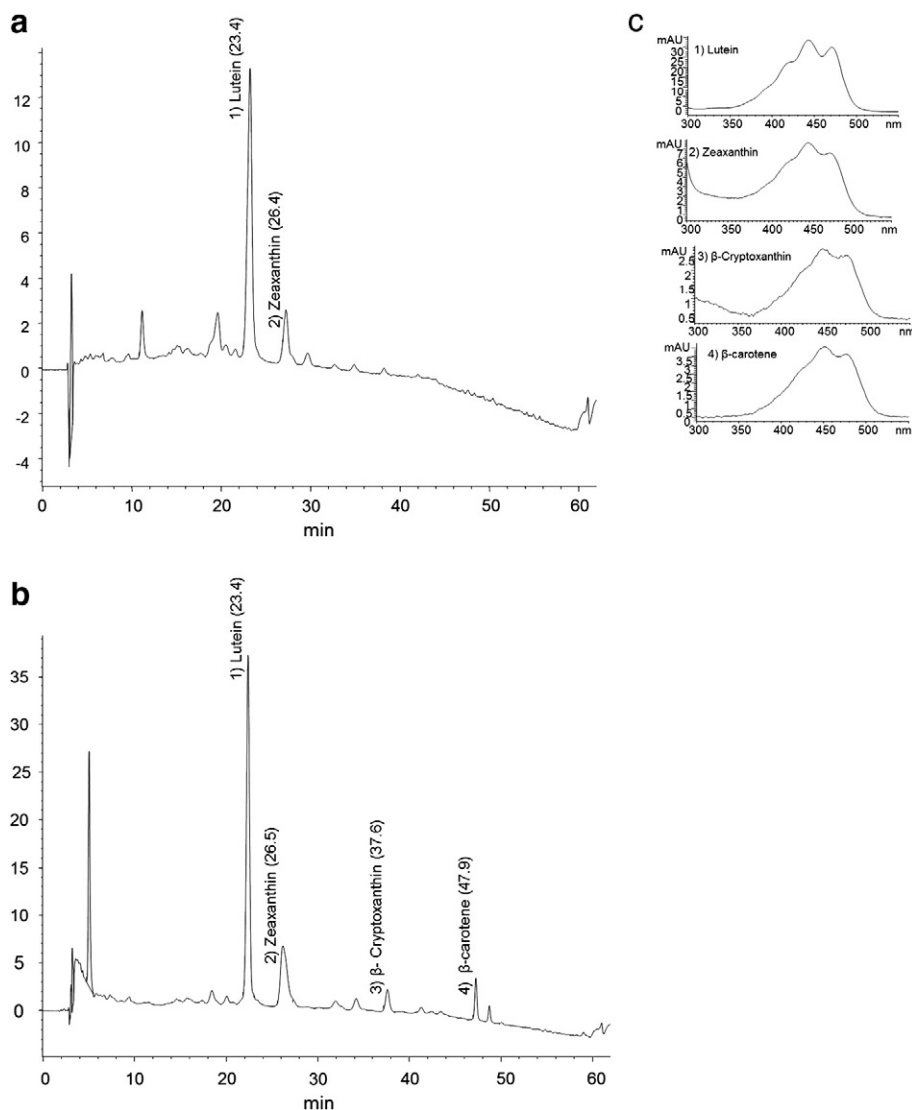
Fig. 6. HPLC profile of total carotenoid extract of callus from *Tagetes erecta* varieties. (a) YF, (b) WF, (c) Absorbance spectra. 1) lutein, 2) zeaxanthin, 3) β-cryptoxanthin and 4) β-carotene.

similar responses on friable callus induction. However they showed differences on carotenoid content (YF, lutein, zeaxanthin; WF, lutein, zeaxanthin; β-cryptoxanthin and β-carotene). Interestingly our results showed that callus from the variety with less pigmented flowers (WF) contained more compounds compared to callus from the pigmented variety (YF), a very important feature and, whereas both varieties induced callus in different proportions, this must be considered for further research work about modification of the carotenoid biosynthesis pathway, on *in vitro* culture cells in order to produce pigments with relevance in the food, pharmacy and feed industries.

Author contributions

Proposed the theoretical frame: PEVE, OPL, AADVM; Conceived and designed the experiments: PEVE; AADVM; Contributed reagents/materials/analysis tools: AJMM, FJH, AADVM; Wrote the paper: IBG, PEVE, OPL, AADVM; Performed the experiments: IBG, AJMM, FJH, AADVM; Analyzed the data: IBG, PEVE, AADVM.

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