# Cyclodextrins Increase Phytosterol and Tocopherol Levels in Suspension Cultured Cells Obtained from Mung Beans and Safflower

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*In this work, suspension-cultured cells of mung beans and safflower were used in order to analyze the effect of methyl jasmonate and/or cyclodextrins, on bioactive compound production such as phytosterols and tocopherols. The results indicated that mung bean suspension-cultured cells produced higher amount of total phytosterols and tocopherols. In particular, mung bean suspension-cultured cells produced almost 220-fold higher levels of tocopherols than safflower suspension-cultured cells in the best conditions. However, while cyclodextrins were able to enhance extracellular production of phytosterols, in the case of tocopherols, they only increased their intracellular accumulation. Our results showed that mung bean cells could be used as a highly efficient system for the production of phytosterols and tocopherols which have a wide range of biological activities.* © 2017 American Institute of Chemical Engineers *Biotechnol. Prog.*, 000:000–000, 2017
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## Introduction

Safflower (*Carthamus tinctorius* L., Asteraceae) is a medicinal plant, which has red and yellow pigments that can be used as food colorants or in the cosmetic industry.[1] Safflower is also able to produce bioactive compounds such as tocopherols and carthamin.[1]

Conversely, mung beans (*Vigna radiata* L., Fabaceae) are an important source of high-added value products such as flavonoids, phenolic acids, phytosterols, and tocopherols,[2] which can be used as antioxidant, antimicrobial, antiinflammatory, antidiabetic, antihypertensive, and antitumor agents.[3,4]

Phytosterols are components of the cellular membranes and they can modulate their fluidity and permeability.[5] The phytosterols reduce the cholesterol levels,[6] they also have antioxidant, anti-inflammatory and antitumoral activities.[7] Moreover, vitamin E represents a family of compounds which is constituted by two chemically different groups, namely tocotrienols and tocopherols. These compounds can act as important antioxidants since they are able to regulate peroxidation reactions and control the production of free radicals within the human body. Due to these important properties they prevent cardiovascular diseases, diabetes, and neurological disorders.[8,9]

The extraction of tocopherols and phytosterols from mung beans and safflower plants is often difficult and expensive. However, we have been able to obtain an alternative system for producing these bioactive compounds using suspension-cultured cells (SCC) treated with $\beta$-cyclodextrins (CDs) and/or methyl jasmonate (MeJA). MeJA is a signal molecule that increases the biosynthesis of bioactive compounds in SCC,[10] while CDs act as elicitors of the biosynthesis of bioactive compounds and also as promoters of the release and accumulation of these metabolites outside the cells.[11–13]

Taking into account all of above, the aim of this study was to evaluate the total production of tocopherols and phytosterols in mung bean and safflower SCC elicited with CDs and/or MeJA.

## Materials and Methods

### *Plant materials*

Calli from mung beans and safflower were established in our laboratory in 2013 from hypocotyl and leaves explants,

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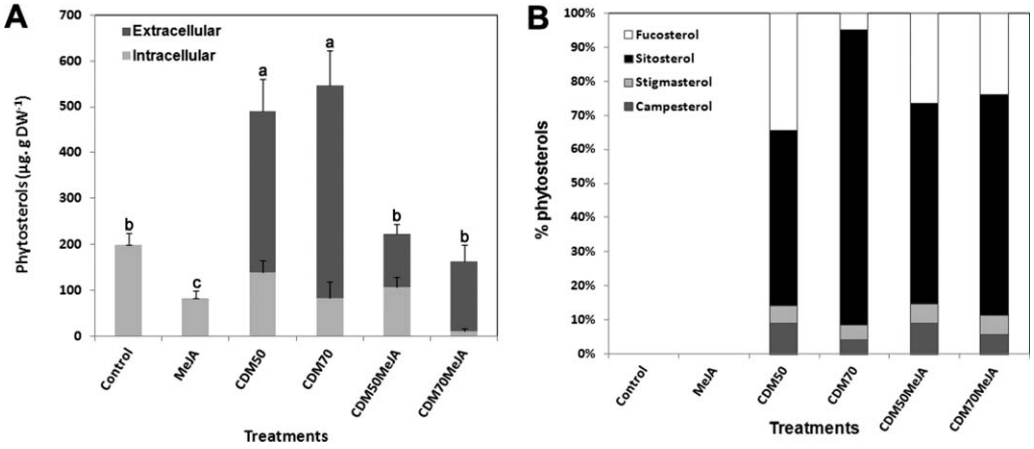


**Figure 1. (A) Effect of 50 or 70 mM CDM and/or 100 μM MeJA individually or in combination on intra and extracellular phytosterol production in mung bean SCC.**

Results were evaluated 144 h after treatments. Values are given as the mean ± SD of three independent experiments with three biological replicates. Treatments significantly different from control were considered after ANOVA with Tukey's multiple comparison tests; $p < 0.05$. (B) Content of each individual phytosterol expressed as a percentage of the total extracellular phytosterol content in sunflower SCC treated with 50 or 70 mM CDM separately or in combination with 100 μM MeJA for 144 h.

respectively. These calli were maintained in Murashige and Skoog medium supplemented with Morel vitamins, 0.25 g $L^{-1}$ casein hydrolysate and 30 g $L^{-1}$ sucrose. In addition, culture medium contained one hormonal dose that in the case of mung beans was 1 mg $L^{-1}$ naphthaleneacetic acid and safflower was 0.55 mg $L^{-1}$ naphthaleneacetic acid and 0.22 mg $L^{-1}$ benciladenine. Mung bean and safflower calli were subcultured on solid culture medium every 21 days. SCC were obtained as described Almagro et al.,[14] and they were routinely maintained by periodical subcultures every 8–11 days.

### *Elicitation of mung bean and safflower suspension cultured cells*

Elicitation experiments were performed using 8-day-old mung bean and 9-day-old safflower SCC. For this, 4 g cells (fresh weight (FW)) were moved into 100 mL flasks and suspended in 20 mL of culture medium which contain CD (50 and 70 mM hydroxypropylated-$\beta$-cyclodextrins (CDH) or methylated-$\beta$-cyclodextrins (CDM)), during 144 h of incubation at 25°C under a 16 h light/8 h dark photoperiod. In addition, 100 μM MeJA was added separately or in combination with CDs.

### *Extraction of phytosterols and tocopherols*

Phytosterols and tocopherols were extracted from both spent media and cells as described by Sabater-Jara and Pedreño[15] and Almagro et al.,[14] respectively. The extracts were filtered through 0.22 μm filters before injecting into a gas chromatography-mass spectrometry (GC/MS) system.

### *Identification and quantification of phytosterols and tocopherols*

The analysis of phytosterols and tocopherols were based on mass spectra obtained from a gas chromatograph Agilent Technologies 6890 Network GS System, equipped with a mass selective detector (Agilent Technologies 5973) as described Almagro et al.[14] The concentrations of phytosterols and tocopherols were estimated on the basis of a standard curve using their respective standard compounds Sigma-Aldrich (Spain).

### *Statistical analysis*

Elicitation experiments were performed in triplicate using three independent experiments with three biological replicates, and all data are given as the mean ± SD. Data were analyzed by ANOVA with Tukey's multiple comparison tests, to confirm data variability and the multiple comparisons of means. All data with $p$ values $\leq 0.05$ were considered statistically significant.

## Results and Discussion

### *Effect of cyclodextrins and methyl jasmonate on phytosterol production in mung bean and safflower SCC*

In this study we have analyzed the effect of two different CD concentrations (50 and 70 mM) in the presence or absence of 100 μM MeJA on phytosterol and tocopherol production in mung bean and safflower SCC during 144 h. In the case of mung bean SCC, the control cells produced high levels of phytosterols (200 μg g dry weight ($DW^{-1}$)) while the levels found with CD or MeJA were 145 or 85 μg g $DW^{-1}$, respectively (Figure 1A). Likewise, the intracellular production levels of phytosterols were substantially lower than those detected in the spent medium when SCC were treated with 50 and 70 mM CDM (370 and 460 μg g $DW^{-1}$, respectively, Figure 1A). In addition, the amount of phytosterols in the combined treatment (CDM and MeJA) were three times lower than those observed in the presence of CDM alone (Figure 1A). The level of each phytosterol in mung bean SCC was expressed as a percentage of the total extracellular phytosterols produced (Figure 1B). The phytosterol found in the highest proportion in the culture medium of cells treated with CDM alone for 144 h, was sitosterol (80% with 70 mM CDM). In addition, the sitosterol content decreased in the presence of CDM + MeJA (Figure 1B).

Conversely, the amount of intracellular phytosterols (Figure 2A) was negligible in safflower SCC. In addition, the higher levels of total phytosterols were detected in 50 mM CDM-treated cells (129 μg g $DW^{-1}$). The addition of CDM and/or MeJA provoked a change in the proportion of phytosterols with respect to control cells, a decrease in sitosterol and campesterol levels being observed (Figure 2B). In agreement with our results, some studies have showed that CDs induced both

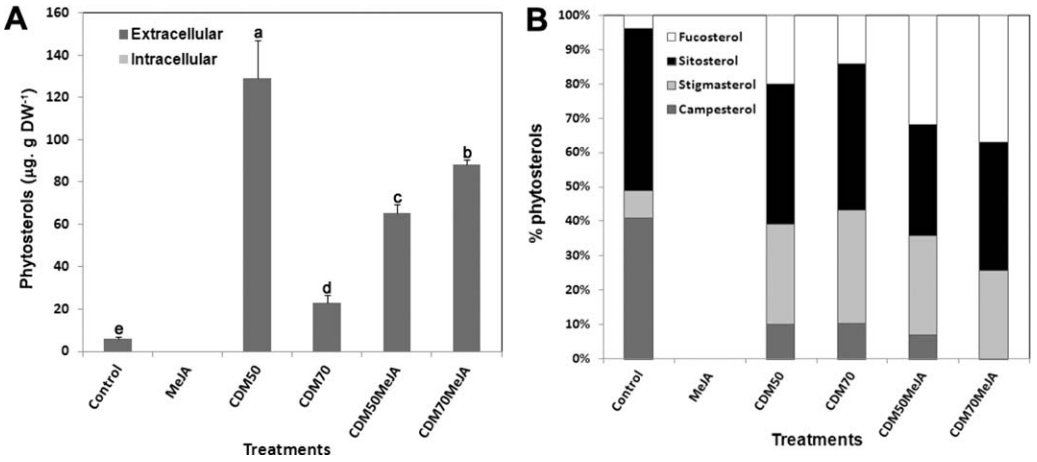


**Figure 2. Effect of 50 or 70 mM CDM and/or 100 μM MeJA individually or in combination on intra and extracellular phytosterol production in safflower SCC. Results were evaluated 144 h after treatments. Values are given as the mean ± SD of three independent experiments with three biological replicates. Treatments significantly different from control were considered after ANOVA with Tukey's multiple comparison tests; $p < 0.05$. (B) Content of each individual phytosterol expressed as a percentage of the total extracellular phytosterol content in mung bean SCC treated with 50 or 70 mM CDM separately or in combination with 100 μM MeJA for 144 h.**

**Table 1. Effect of 50 or 70 mM CDM or CDH on Intra- and Extracellular Tocopherol Production in Mung Bean and Safflower SCC**

| | Mung beans | | Safflower | |
|---|---|---|---|---|
| | μg g $DW^{-1}$ (intracellular) | μg g $DW^{-1}$ (extracellular) | μg g $DW^{-1}$ (intracellular) | μg g $DW^{-1}$ (extracellular) |
| Control | 220.5 ± 24.1a | 0.13 ± 0.05c | 0.05 ± 0.03b | 0.76 ± 0.0008a |
| CDM 50 mM | 68.1 ± 25.6c | 1.42 ± 0.18a | 0.14 ± 0.09b | 0.13 ± 0.01c |
| CDM 70 mM | 110.0 ± 1.8b | 0.83 ± 0.07b | 0.2 ± 0.07a | 0.05 ± 0.003d |
| CDH 50 mM | 85.9 ± 14.6c | 0.75 ± 0.08b | 0.27 ± 0.01a | 0.72 ± 0.03a |
| CDH 70 mM | 177.5 ± 22.2a | 1.31 ± 0.15a | 0.27 ± 0.01a | 0.45 ± 0.02b |

Results were evaluated 144 h after treatments. Vales are given as the mean ± SD of three experiment with three replicates. Treatments significantly different from control were considered after ANOVA with Tukey's multiple comparison tests; $p < 0.05$.

the production as well as extracellular accumulation of bioactive compounds like taraxasterol,[16] resveratrol,[11] and phytosterol[15] in SCC. In fact, Briceño et al.,[16] Sabater-Jara and Pedreño,[15] and Almagro et al.[14] also observed that the addition of CDs to tomato, carrot and flax SCC enhanced the levels of phytosterols in the spent medium. Moreover, our results also agreed with those found by Bonfill et al.[17] who showed that the levels of phytosterols decreased when MeJA was added to *Centella asiatica* SCC. Mangas et al.[18] also observed that 100 μM MeJA reduced the levels of phytosterol *in vitro*plants of *Centella asiatica, Galphimia glauca*, and *Ruscus aculeatus*.[18] The decrease of phytosterols observed after the treatment with MeJA suggested a competition between the different pathways conducting the carbon flow through the metabolite biosynthetic pathways in plants.

### *Effect of cyclodextrins and methyl jasmonate on tocopherol production in mung bean and safflower SCC*

The elicitation with CDM or CDH (50 or 70 mM) provoked a decrease of tocopherol production in mung bean SCC (Table 1) as compared to control SCC. In fact, the highest tocopherol levels were detected inside the cells in all treatments, and only a low concentration of tocopherols were observed in the culture medium in CD-treated cells (Table 1). A similar trend was found in safflower SCC (Table 1). Moreover, the joint action of CDs and MeJA in both cell lines provoked a decrease of total tocopherol levels compared to CD-treated cells (data not shown).

The results indicated that mung bean and safflower SCC have significantly different levels of tocopherols. In particular, mung bean SCC produce almost 290-fold higher levels of tocopherols than safflower SCC in the best conditions. Moreover, the levels of tocopherols are similar to those found in in other SCC. In fact, photomixotrophic SCC of sunflower were able to produce 77 μg g $DW^{-1}$.[19] Likewise, the addition of precursors like homogentisic acid to sunflower SCC enhanced the levels of tocopherol (24 μg g $FW^{-1}$).[20] More recently, Almagro et al.[14] also showed that CDs were able to enhance the levels of tocopherols in flax SCC.

## Conclusion

In conclusion, CDs enhance the extracellular levels of phytosterols in safflower and mung bean SCC, being the total levels of tocopherols lower in SCC elicited with CDs than in control SCC. In addition, mung bean SCC produce greater levels of these compounds than safflower SCC. Therefore, mung bean SCC could be used for the biosynthesis of phytosterols in the presence of CDs or tocopherols in the absence of CDs.

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