Comparison of Bioactive Secondary Metabolites in Experimental and Natural Populations of Wild Tomatillos, *Physalis longifolia* Nutt.

Kelly Kindscher, Cong-Mei Cao, Robert J. Gallagher, Huaping Zhang, Quinn Long, Lauren Service, Leanne M. Martin, and Barbara N. Timmermann

Research

Abstract

We conducted a field experiment to determine the effects of mulch, fertilizer, and mycelium on biomass and important secondary metabolite concentrations in the edible and medicinal plant *Physalis longifolia* Nutt., with the hypothesis that increased plant stress (i.e., no mulch, fertilizer, or mycelium) would decrease biomass production and increase secondary compound content. Experimental cultivated plots and natural populations of *P. longifolia* were evaluated for the abundance of major bioactive withanolides previously isolated from the species: withalongolide A (1), withaferin A (2), and withalongolide B (3). Results indicated negligible differences between experimental treatments in biomass yield and withanolide abundance. However, withanolide concentrations from wild populations varied considerably with some being much higher than the source population used in the experiment. These results suggest that variation in secondary compound concentrations among wild populations is an important consideration when selecting source material for the cultivation of medicinal plants.

Introduction

The wild tomatillo, *Physalis longifolia* Nutt., is a small herbaceous perennial plant found in weedy, disturbed habitats and crop fields across much of North America from southern Canada to northern Mexico. This and related *Physalis* species have been important wild-harvested edible and medicinal plants in the Midwest, Great Plains, and Southwest U.S.A. We have documented 23 tribes in the U.S. Southwest and Midwest who use or have used the edible portions of *P. longifolia* and other *Physalis* species as important foods or medicines (Kindscher *et al*. 2012). These species were eaten raw, prepared as a green sauce, and dried as pats or cakes. The Zuni dried and ground the berries to produce a meal for making bread (Hough 1898), and they also boiled the ripe fruits with onions, chili, and coriander seeds to make a flavorful tomatillo sauce (Castetter 1935). The practice of making sauce among the Zuni and eating wild tomatillos in the Southwest continues through today (Edaakie 1999), as we have also observed that *P. longifolia* is encouraged to grow as a useful weed in Jemez pueblo corn fields. The ethnobotanist Melvin Gilmore (1913) reported that *Physalis* fruit were also made into a sauce for food by the Omaha, Ponca, Dakota, and Pawnee, and when there was sufficient quantity, the fruit were dried for the winter. Their importance is also substantiated by the frequent discovery of *Physalis* seeds at archeological sites (Kindscher *et al*. 2012). In addition, *Physalis* species, including *P. longifolia*, have been used as medicine, with the Omaha and Ponca using the “crooked medicine” to treat headache

Correspondence

Kelly Kindscher, Leanne M. Martin, Lauren Service, Kansas Biological Survey, University of Kansas, Lawrence, Kansas, U.S.A.
Cong-Mei Cao, Robert J. Gallagher, Huaping Zhang, Barbara N. Timmermann, Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas, U.S.A.
Quinn Long, Missouri Botanical Garden, Saint Louis, Missouri, U.S.A.

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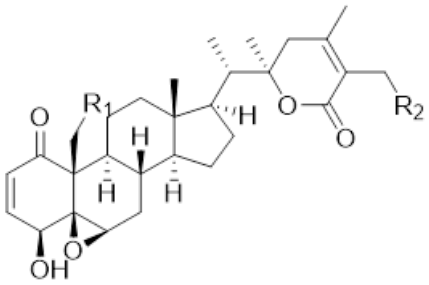
and stomach trouble and to dress wounds (Gilmore 1977, Kindscher *et al*. 2012).

Our interest in wild tomatillos stems from our development of a Native Medicinal Plant Research Program at the University of Kansas where we have used ethnobotanical information from our database to select native species in Kansas (Kindscher *et al*. 2013) and the Great Plains for useful chemical compounds. Through that process, we made use of our university's High-Throughput Screening Laboratory to screen over 200 species of ethnobotanical importance. Among our findings, we discovered that the species with the highest antioxidant content was *Physalis longifolia*. This result may explain the interest in the fruit as food and encouraged us to continue exploring this species for its anti-cancer activity and its interesting chemistry.

In the first phytochemical study of this species we isolated the major constituents withalongolide A (1), withaferin A (2), and withalongolide B (3), in conjunction with 22 other withanolides (Zhang *et al*. 2011) (Figure 1). Withanolides 1−3 exhibited promising antiproliferative activity against human head and neck squamous cell carcinoma (JMAR and MDA-1986) and melanoma (B16F10 and SKMEL-28) cell lines, while maintaining comparatively low toxicity in normal fetal fibroblast (MRC-5) cells (Samadi *et al*. 2012, Zhang *et al*. 2012). As part of our ongoing research, we established a comprehensive HPLC/PDA (high performance liquid chromatography with photo diode array detector) method to determine the presence and concentration of withanolides 1−3 in *P. longifolia* collections from both wild and cultivated origins. Through this chemical profiling method we have the ability to distinguish potential differences in secondary metabolite concentrations among wild and cultivated populations.



		R_1	R_2
1	Withalongolide A	-OH	-OH
2	Withaferin A	-H	-OH
3	Withalongolide B	-OH	-H

Figure 1. Structures of the withanolides (1–3) examined in this study.

Due to the potential importance of this species for medicinal use, we conducted an experiment to determine how alteration of growing conditions would affect withanolide concentrations. Previous research has shown that although plant stress generally increases secondary metabolite concentrations, numerous contributing factors may influence this response (Figueiredo *et al*. 2008, Selmar & Kleinwächter 2013) such as cultivar selection, developmental stage, fertilization rates, soil pH, plant competition, and season of harvest, as well as climatic factors including CO_2 concentration, water availability, and light duration, intensity, and quantity (Björkman *et al*. 2011). As shown in these studies, variation in many of these environmental factors can induce or ameliorate plant stress in both wild and cultivated populations. Another study from a drought-prone region has shown that guayule (*Parthenium argentatum* A.Gray) generally produces a higher content of secondary metabolites (latex) when plants are moderately water-stressed (Veatch-Blohm *et al*. 2006).

Plants from wild sources often contain higher concentrations of secondary metabolites. For example, wild raspberries (*Rubus idaeus* L.) (Çekiç & Özgen 2010), wild fennel (*Foeniculum vulgare* Mill.) seeds, and wild laurel (*Laurus nobilis* L.) leaves (Conforti *et al*. 2006) have all been shown to have higher antioxidant capacity than popular cultivated crops. Lower secondary compound concentrations observed in cultivated plant material may reflect crop cultivar selection and production practices which favor total yield and volume without consideration of secondary metabolites, specifically the use of fertilizers and irrigation to moderate naturally occurring environmental stress. Our research hypothesis was that alleviating stress on plants by adding mulch and increasing nutrient availability would increase biomass, whereas greater exposure to stress by not mulching or not increasing nutrients would result in higher levels of secondary metabolites.

Materials and Methods

Plant materials, experimental design, and data collection

We collected the aerial parts and fruit of *P. longifolia* from a wild population in Stafford County in central Kansas, U.S.A. A voucher specimen was deposited in the R.L. McGregor Herbarium—KANU—at the University of Kansas, Lawrence, Kansas (Hillary Loring 4124). Seeds from these fruit were grown into two-month-old plants at the University of Kansas greenhouse and transplanted in May 2011 into our Native Medicinal Plant Research garden, which is located in the prime agricultural floodplain of the Kaw River in Rossville silt loam soil (USDA, NRCS 2013) near Lawrence, Kansas (see: http://nativeplants.ku.edu/about/medicinal-plant-garden). Throughout the experiment, we weeded the experimental plots but only watered sufficiently to establish plants after transplanting them from the greenhouse. Our research site has a temper-

ate climate and average rainfall of 97 cm (38 inches) per year, although precipitation during the year of our 2011 field experiment was only 88 cm. Furthermore, from 2 August 2011 through our October harvest, Douglas County, Kansas, was classified as in drought (U.S. Drought Monitor 2013).

We had four cultivated treatment types: mulch (of local wood chips), mulch and mycelia, mulch and fertilizer, and control (unmulched). Mulch was applied to cover the ground thoroughly to ameliorate the effects of drought and thus reduce plant stress. Fertilizer was applied at a rate of 67 kg per ha (60 pounds per acre) of ammonium nitrate, which is the amount that Kansas wheat crops often receive. We included a treatment using the mycelia of wine-capped stropharia (*Stropharia rugosoannulata* Farl. ex Murrill) with woodchips to investigate whether mycelia, while decomposing the woodchips, might also increase nutrient availability (Stamets 2005). We predicted that the unmulched control group would experience greater moisture- and nutrient-limitation than other treatments, resulting in the highest secondary metabolite concentration within the experiment.

The experiment was a fully randomized design in which 24 plants were transplanted into each plot, with five replicate plots for each of the four treatment types (Figure 2). In early October of 2011 we harvested fruits and total above-ground biomass from each plot. To facilitate comparisons with wild-collected plant material, fruit were separated from the harvested plant material, and HPLC analysis was conducted on extracts from the dried, coarsely-ground, above-ground vegetative biomass. In addition, we collected above-ground vegetative biomass from four wild *P. longifolia* populations in different counties in Kansas. The materials were dried, ground, stored, and analyzed using the same methods as outlined above for the cultivated plant material (KANU vouchers: Loring 3583, 4124, 4107, and 4095). The wild collections reflect an east-to-west gradient across Kansas (Figure 3).

Medicinal chemistry research techniques used for withanolide detection

Chemical profiling was conducted by HPLC analysis performed on an Agilent 1200 series system (Agilent Technologies, U.S.A.) equipped with a quaternary pump, an auto sampler, and a PDA and connected to ChemStation software. Chromatographic separation was performed using an IRIS IProSIL120-5 C18 AQ column (4.6 mm × 250 mm, 5.0 µm). HPLC-grade acetonitrile and analytical grade solvents were purchased from Fisher Scientific Co. (Fair Lawn, New Jersey, U.S.A.). HPLC-grade water was prepared using a Millipore Milli-Q A10 system (Millipore Corp., Bedford, Massachusetts, U.S.A.). Withanolide standards [withalongolide A (1), withaferin A (2), and withalongolide B (3)] used in these experiments were purified from isolates obtained in our previous phytochemical investigation of *P. longifolia* (Zhang *et al*. 2011). The structures of the three withanolides (Figure 1) were identi-

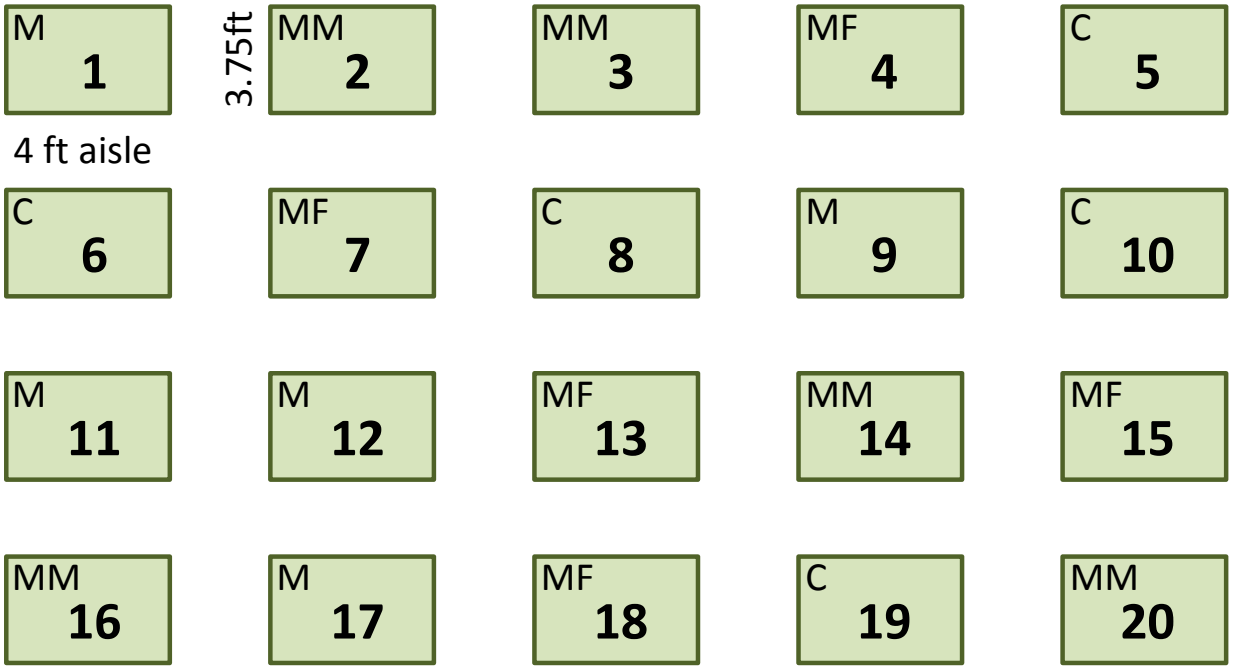


Figure 2. Wild tomatillo (*Physalis longifolia* Nutt.) experimental plot design, with five plots for each of four treatments: mulch only (**M**), mulch with mycelia added (**MM**), mulch and fertilizer (**MF**), control (**C**). Each plot is 152 × 213 cm (5 × 7 ft) with 24 (4 rows of 6) plants. Aisles between rows are 121 cm (4 ft) and 114 cm (3.75 ft) as shown in the diagram.

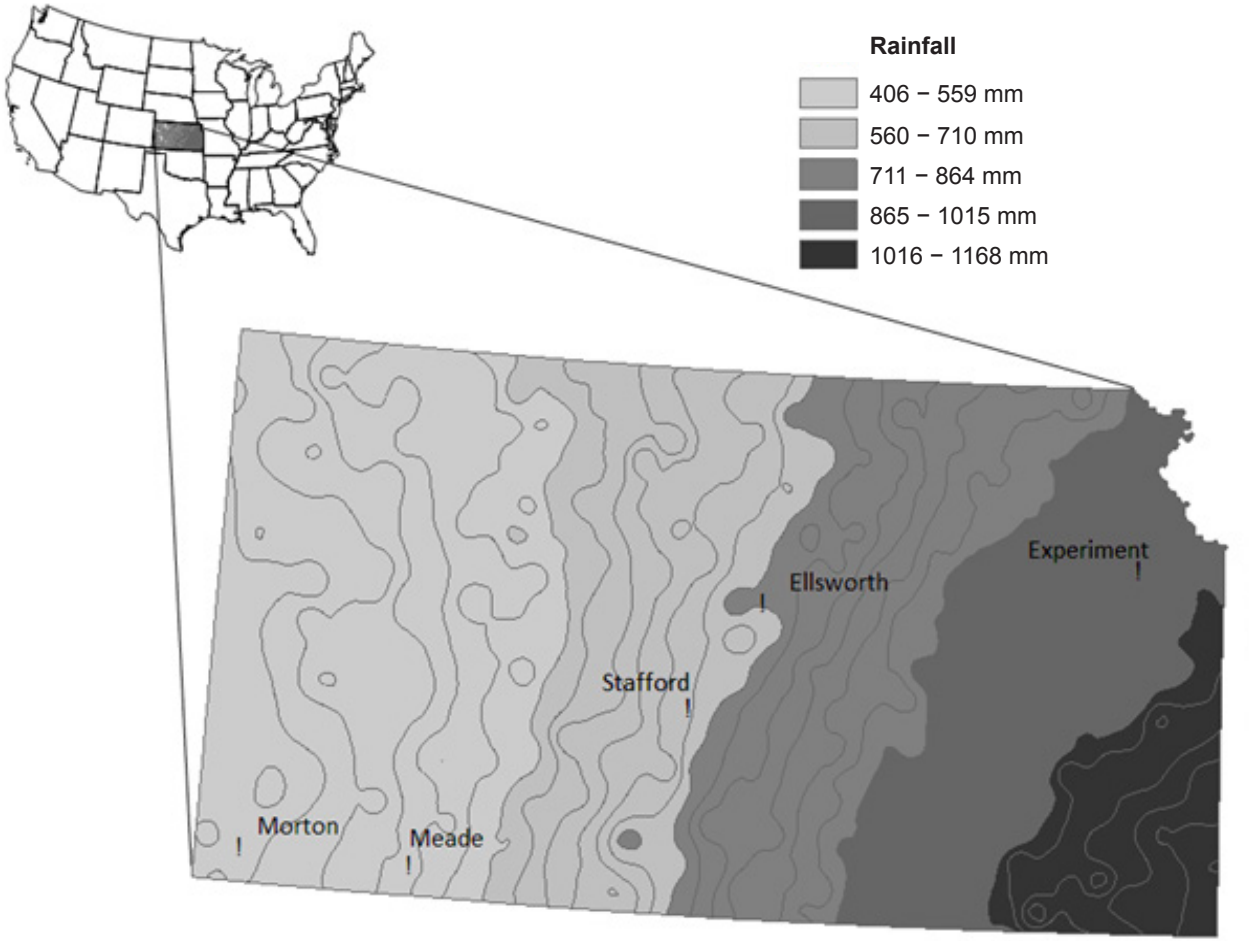


Figure 3. Locations of wild *Physalis longifolia* Nutt. collections and the experimental study site in the context of the rainfall gradient from west to east across Kansas and the Great Plains, U.S.A.

fied by UV, MS, 1H, and 13C NMR spectral data (Zhang *et al*. 2011). HPLC analysis determined that these compounds were greater than 97% pure. The newly established HPLC/PDA method was applied for simultaneous detection of withanolides 1–3 in five batches (the experimental replicates) of the four treatments collected from experimental plots. The withanolide concentrations of each collection, including the wild populations, were calculated from an average of triplicate extraction measurements (i.e., using three subsamples of homogenized material per collection).

Data analysis and statistics

Analyses were conducted using a Kruskal-Wallis test in conjunction with a series of non-parametric Mann-Whitney U tests to determine differences between withanolide content among cultivated treatments. To correct for the inaccuracies of conducting multiple comparisons, a conservative Bonferonni correction (Dunn 1964) was employed to determine when $p < 0.05$ between all groups and treatments. We were unable to statistically compare withanolide concentrations of wild populations to each other or to cultivated populations because we had only one average value per wild population (calculated from three sub-samples of homogenized plant material) and our cultivated plants were propagated from only one population. Analyses were conducted using SPSS (2011).

Results and Discussion

Above-ground biomass production of *P. longifolia* was not statistically different between the experimental treatments (mulch; mulch and mycelia; mulch and fertilizer; and unmulched control) (Kruskal-Wallis test; $p = 0.898$; average biomass = 5.31 kg; standard deviation = 0.90 kg). In observation of the plots, the plants appeared fairly uniform in stature and without any apparent benefits of fertilizer or mycelia. The withanolide concentrations of the experimental treatment collections and the wild collections are presented in Table 1. All experimental treatments were grown from seed of the Stafford County collection, which surprisingly contained the lowest withanolide content of all wild collections examined.

Table 1. Average concentrations of withalongolide A (1), withaferin A (2), and withalongolide B (3) calculated from three repeated tests of a single bulk collection per population of *Physalis longifolia* Nutt. *The cultivated material was grown in Douglas County, Kansas, U.S.A.

Location County, State	Collection date (mm/dd/yyyy)	Withanolide concentration (µg - g-1)		
		1	2	3
Cultivated*	10/03/2011	504.56	424.13	228.71
Ellsworth, Kansas	08/09/2009	2272.90	1510.36	582.41
Meade, Kansas	08/01/2010	1058.82	1295.52	510.96
Morton, Kansas	08/09/2010	764.16	1058.59	350.45
Stafford, Kansas	09/02/2010	635.67	704.36	325.11

Withanolide concentrations in cultivated P. longifolia collections

All four treatment types demonstrated similar withanolide abundance trends where withanolide 1 was the most abundant compound followed by 2 and 3 (Figure 4). Comparing cultivated collections, the mulch and mycelia treatment produced the highest concentrations of both withanolides 1 and 3, yet a lower withanolide of 2 concentration compared to the mulch and fertilizer treatment. However, differences in specific withanolide concentrations in response to treatments were not statistically significant ($p > 0.05$) for each of the three withanolide comparisons (Figure 4).

Withanolide concentrations in wild P. longifolia collections

The wild collection sample from Ellsworth County, Kansas, contained the highest overall total withanolide concentration and differed in the relative concentrations of constituent withanolides compared to the other wild populations (Figures 5, 6). The Ellsworth County collection displayed the same withanolide trend observed in the treatments, where withanolide 1 was the most abundant compound followed by 2 and then 3. The other three wild sam-

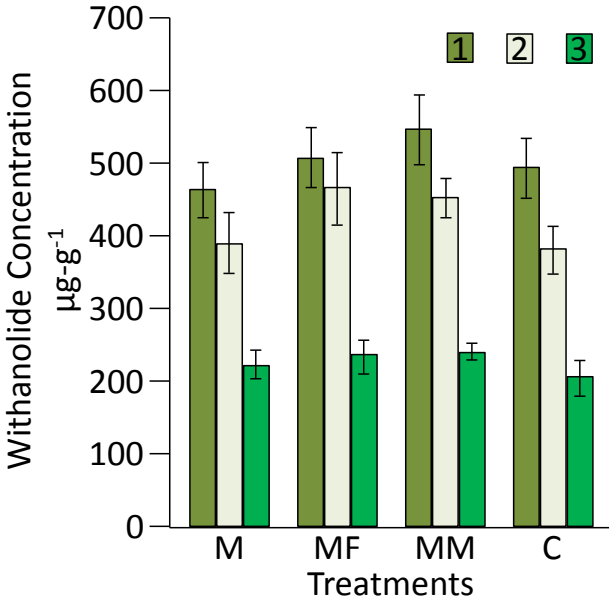


Figure 4. Average concentrations (µg - g^{-1} +/- 1 SE) of withanolides (1–3) in treatment types: mulch (**M**), mulch and fertilizer (**MF**), mulch and mycelia (**MM**), and control/no mulch (**C**) in cultivated *Physalis longifolia* Nutt. Error bars indicate variation among average withanolide values for replicate plots.

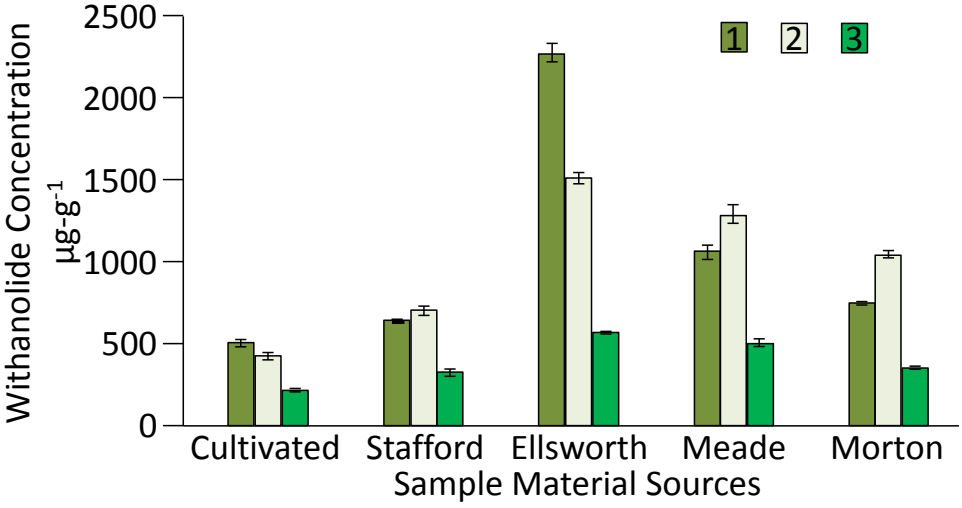


Figure 5. Comparison of withanolide (1–3) concentrations (µg - g^{-1} +/- 1 SE) in cultivated and wild populations of *Physalis longifolia* Nutt. Error bars for wild populations indicate the amount of variation found within the three subsamples tested for each location, whereas the error bars for the composite of cultivated material represents variation among average withanolide values for each experimental treatment.

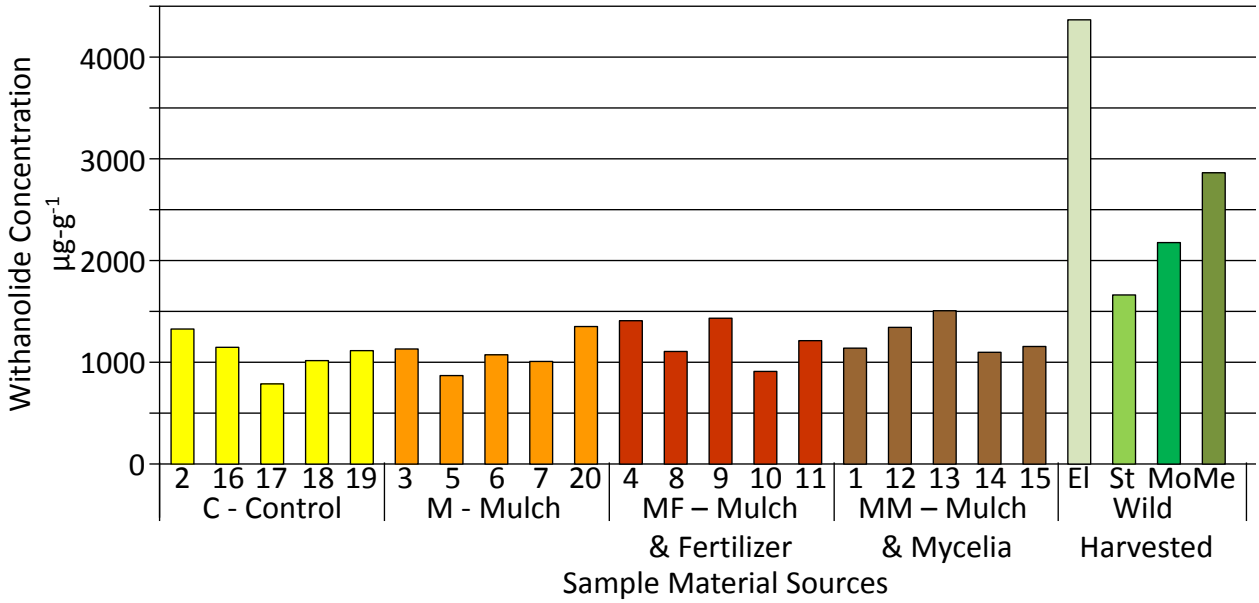


Figure 6. Total withanolide abundance by treatment (withanolide 1+2+3) with five replicate plots (with plot numbers) for each cultivated treatment and four wild harvested samples of *Physalis longifolia* Nutt., where wild harvested samples are El = Ellsworth County, St = Stafford County, Mo = Morton County, and Me = Meade County (all counties from Kansas, U.S.A.).

ples showed lower concentrations of each withanolide, but exhibited different patterns of relative abundance among withanolides.

Comparison of withanolide concentrations in cultivated and wild P. longifolia populations

Even though there were no significant differences in withanolide concentrations among the cultivated treatments, there were pronounced differences in withanolide concentration among wild populations. These results suggest that our experimental treatments were not able to generate the high levels of variation exhibited in wild populations, and that the mechanisms generating this variation among wild populations require further examination. The composite sample from the Stafford County collection exhibited lower withanolide concentrations than other wild collections, which may have influenced the low withanolide concentrations observed given that the cultivated material was sourced solely from this collection. Other populations, most notably the Ellsworth County collection, exhibited strongly elevated withanolide concentrations, suggesting that this population is a more desirable seed source for plant materials. It should be noted that we have recently propagated this population from root cuttings collected during the spring, which is more efficient than collecting mature seeds in the fall and propagating plant material from seed. These efforts to refine propagation protocols with wild-sourced material will facilitate future experimentation.

All wild collections were harvested when the plants were flowering and fruiting in August and September of either 2009 or 2010. There are no obvious differences due to date of harvest. One might expect a gradient of higher secondary metabolite concentrations moving westward along a longitudinal gradient given that rainfall is much reduced as one moves west across the Great Plains, but this was not observed among the populations sampled. The variation of the withanolide concentrations may be due to climatic factors prior to when the plant material was harvested or other environmental influences not taken into account. For example, annual average rainfall in Figure 3 does not occur for drought conditions during the year or season when the compounds are being produced. Genetic variability among populations, the age of perennial plants, and differing soils may also have affected withanolide concentrations.

Conclusions

Our comprehensive HPLC/PDA method successfully determined the presence and concentration of the medicinally-important withanolides, 1–3, in wild and cultivated *P. longifolia* collections. There were insignificant biomass and withanolide yield differences observed between experimental treatments. Given that biomass production was unaffected by cultivation treatments, there is no evidence to suggest that the treatments induced or alleviated plant stress.

The cultivated plant material exhibited only minor differences in withanolide concentrations among treatments. Although the mulch-plus-mycelia and mulch-plus-fertilizer treatments exhibited slightly higher withanolide concentrations relative to other treatments, these differences were not significant. Interestingly, these trends are contrary to our expectation that treatments that reduce plant stress would result in lower secondary metabolite production. Accordingly, we can neither reject nor confirm the hypothesis that added stress would increase the content of secondary metabolites. Given larger sample sizes in future work, it is possible that these trends could provide insights for utilizing different cultivation techniques to increase withanolide concentrations of cultivated *P. longifolia*.

Although the cultivated material did exhibit slightly lower withanolide concentration relative to the wild collection from which it was propagated, these differences are minor compared to the magnitude of differences among wild populations. Although our data do not provide evidence of cultivation causing a reduction in secondary compound concentration, our observations do suggest that the low withanolide concentration of a wild source population can be preserved in cultivation. Given the substantial variation in secondary compound concentration among wild populations, this suggests that such differences among wild populations may be an important consideration when selecting source material for the cultivation of medicinal plants.

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