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Full Length Research Paper

Impact of different colored shade polyethylene and maturation on development of flavonoids and phenolic acids in flowers of *Chrysanthemum indicum* L.

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***Chrysanthemum indicum* L. is a significant medicinal plant, which flowers (capitula) are usually used as Chinese medicine. The phytochemical test analyzed the regularity of biosynthesis and degradation of flavonoids and phenolic acids in flowers which grown under colored polyethylene and harvested from five flowering stages. Dry weight of one thousand buds or flower heads from different maturation stages and total number of flowers from individual plant were evaluated as biomass yield. The results indicated that the total number of buds and flower heads of individual plant grown under red polyethylene film was substantially higher than other treatments (*P*<0.01), as well as accumulated more quercetin, apigenin and chlorogenic acid, but gave poor content of luteolin and caffeic acid. Comparing with plants grown in open condition, blue polyethylene has the lowest mean weight of one thousand buds or flower heads from different harvesting stages, but it has potentials to increase the total number of buds and flower heads from individual plant and the yield of bioactive compounds total flavonoids, quercetin, apigenin, acacetin, linarin, chlorogenic acid and caffeic acid, except luteolin.**

Key word: *Chrysanthemum indicum* L., colored polyethylene, maturation, flavonoids, phenolic acids.

INTRODUCTION

Chrysanthemum indicum L., spreading widely in China, is a well-known herb with small yellow flowers (Zhu et al., 2005). Its flower (capitulum) is considered as a traditional folk drug (Matsuda et al., 2002; Cheng et al., 2005) and regarded as a conventional endemic vegetable (Zhao et al., 2007). Previous studies have reported that flavonoids and phenolic acids are ubiquitously distributed secondary metabolites in plants (Tanaka and Ohmiya, 2008; Fiamegos et al., 2004). Phytochemical profile of this plant had shown the presence of flavonoids and phenolic compounds (Yoshikawa et al., 1999). A plant is a complex and ever active chemical factory that produces a wide range of chemical moieties (Dweck, 2009). Light is an important physical or ecological factor, which influences the growth and the formation of primary and secondary metabolites (Yu et al., 2005). It is hypothesized that differences in environmental factors could affect the development of *C. indicum*, moreover, light environment may play a pivotal role in biosynthesis and degradation of secondary metabolites. Khandaker et al. (2010) lectured that different colors of polyethylene shade could have ability to reflect different spectra of the visible light that may affect crop development and yield. When other conditions were the same, would colored polyethylene alter the growth and the concentrations of the two main active components in capitulum by manipulating the light quality around the herb? Little information is available. The objective of the current survey is to clarify how flavonoids and phenolic acids changes in capitulum from visible immature buds to blossomy inflorescence matures affected by light quality regulated cultivation using different colored shade polyethylene.

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MATERIALS AND METHODS

Chemicals

Standards of Rutin (≥95%), Luteolin (≥98%), Quercetin (≥98%),

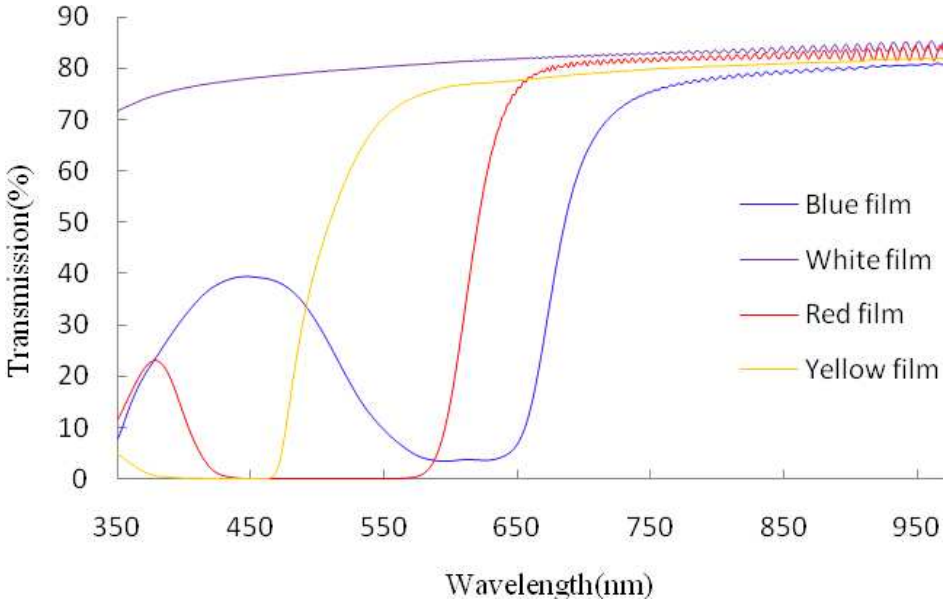

Figure 1. Spectral distribution of the transmission spectra of colored plastic films.

Table 1. Illuminances and illumination percentages of colored plastic films.

Treatment	Illuminance (lx)	Range (lx)	Ratio (%)
Control	75250±173	75100-75400	100.00
White film	66750±1767	67100-69100	88.70
Yellow film	53575±5885	49400-62300	71.20
Red film	12167±2059	10300-15600	18.07
Blue film	7640±817	6600-8600	10.63

Data are expressed as the mean ± standard deviation and are the mean of nine replicates.

Acacetin (≥97%), Apigenin (≥95%), Chlorogenic acid (≥95%), Caffeic acid (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA); Linarin (≥98.3%) was obtained from National Institute for the control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC grade solvents) was obtained from Merck (KGaA, Darmstadt, Germany). Acetonitrile (HPLC grade solvents) was supplied by Honeywell (Burdick and Jackson, Muskegon, MI, USA). All other chemicals and reagents were of analytical grade.

Material and treatments

Characterization of colored polyethylene films

Transmission spectra (Figure 1) of colored polyethylene films (produced by WeiKang Ltd., Shanghai, China) were analyzed by UV-Vis spectrophotometer (PerkinElmer, Lambda25), scanning from 1000 to 350 nm, with a step of 2 nm. Illuminances (Table 1) of colored films were estimated by luminometer (Testo, 545) at later-morning and early-afternoon on three consecutive days. Transmission percentages of each film were calculated and presented in Table 1.

Cultivated treatment and sample preparation

C. indicum of natural populations used in this study were authenticated and collected in October 2007 from barren mountain areas of Nanjing. In the next spring, Herbs were individually planted in pots (diameter 25 cm × height 25 cm) in standard peat mixture and grown with general maintenance. Plants grown in open condition (non-shade frame) were used as control. Other herbs were subjected to grown under different colored shade polyethylene (White, Yellow, Red, and Blue) in whole life.

Growth chambers (length 200 cm × width 100 cm × height 150 cm) covered with colored polyethylene was applied. As the light transmittance was different for each colored polyethylene, shading methods were applied to ensure equal light quantity of each film according to blue film. Humidity, temperature, photoperiod and other environmental conditions were all in accord with the nature throughout the study period. The experiments were conducted at the roof-top garden of Life Sciences building of Nanjing Agricultural University. Buds or flowers in different maturation were harvested from five flowering stages: Stage 1: both the ray and the disc florets unopened; Stage 2: the ray florets loosened 30% and the disc florets unopened; Stage 3: the ray florets loosened 50% and the disc florets

loosened 30%; Stage 4: the ray florets loosened 70% and the disc florets loosened 50%; Stage 5: both the ray and the disc florets fully opened.

All buds or flowers were transported to the lab within 30 min after harvest. Samples were air-dried in an oven at 40℃ for 16 h, and then the dried samples were stored in a refrigerator at -20℃ for further studies. Total number of buds and flowers from individual plant and dry weight of one thousand buds or flower heads fromdifferent stages were determined as the dominating parameter for biomass yield evaluation.

Extraction procedure

The dried flowers were ground with a blender and sieved through a 100-mesh screen to obtain flower powder which was sealed in tight polyester bottles and stored at -20℃. Herbal powders were ultrasonic extracted at room temperature in methanol (10 ml) for 1 h and centrifuged at 6500 rpm for 10 min at 4℃. The sa mple was re-extracted three times. The supernatants were combined and made up to 50 ml with methanol, then filtered through a 0.45 µm Nylon membrane filter (Millipore) as test solutions for phytochemical analysis.

Flavonoids quantification

Total flavonoids

For total flavonoid examination, the method of Jia et al. (1999) was modified for using. In brief, exactly 0.3 ml of 5% $NaNO_2$ was added to a 1 ml test solution in a 10 ml volumetric flask and the mixture was kept for 6 min at room temperature.

Addition of 0.3 ml of 10% $Al(NO_3)_3$ to the mixture, which was incubated for 6 min again, was followed by addition of 4 ml of 1 N NaOH and of methanol, up to volume. After incubating for 15 min at room temperature for color development, absorbance at 510 nm was measured. Total flavonoid content was expressed as rutin equivalents.

Linarin

Linarin analysis was based on the method from Chinese Pharmacopoeia (volume I) (Chinese Pharmacopoeia Committee, 2005), using a Shimadzu Prominence LC (LC-20AT, SPD-20A) (Shimadzu) with a C18 column (Shim-pack VP-ODS, 4.6 × 250 mm, 5 µm). A mobile phase consisted of methanol: water: glacial acetic acid (26: 23: 1, v/v/v) was used. Detection was achieved at 334 nm, the flow rate was 1.0 mL/min. 20 µL aliquot of test solutions was directly injected. The quantification was performed using commercial external standard, which was passed through the same process.

Quercetin, luteolin, apigenin and acacetin

Flavonoids usually occur as glycosides and aglycones in plant tissue (Saxena et al., 2009). Test solutions were subjected to acidic hydrolysis (2 N HCl) to break the glycoside linkages, according to the methods reported by Sakakibara et al. (2003). Conditions for the HPLC separations were those reported by Shen et al. (2010a), A 20 µl aliquot of hydrolysates was injected into an Agilent 1120 Compact LC (G4288A) (Agilent Technologies) with a C18 column (Agilent Zorbax Eclipse XDB, 4.6 × 250 mm, 5 µm).

The mobile phase consisted of methanol (A) and 0.2% phosphoric acid in water (B). The linear gradients used at a flow rate of 1.0 ml/min were 47% A, 53% B (0 to 20 min), 47 to 70% A, 53 to 30% B (21 to 30 min), 70% A, 30% B (31 to 40 min), 70 to 100% A, 30 to 0% B (41 to 45 min) and 100% A, 0% B (46 to 50 min). The column was then returned to the initial conditions and equilibrated for 15 min. The peaks were detected at 350 nm. Samples were injected and quantified using external standards of quercetin, luteolin, apigenin, acacetin.

Phenolic acids quantification

The method of Shen et al. (2010b) was used for chlorogenic acid and caffeic acid quantization. All analyses were performed on an ÄKTA purifier (GE Healthcare) using Shim-pack VP-ODS C18 column (4.6 × 250 mm, 5 µm). A 100 µl aliquot of test solutions was directly injected, then eluted with a solvent system of acetronitrile : water (81: 19, v/v), the water contained 0.5% phosphoric acid. The flow rate was 1.0 ml/min. The 326 nm UV absorbance was used for monitoring. External standards of chlorogenic acid and caffeic acid were applied to quantification.

Statistical analysis

Experimental results were means ± standard deviation of three parallel measurements. The data were analyzed by an analysis of variance (ANOVA) and the means separated by Duncan's multiple range tests at the 1% level (Microsoft Office Excel 2007 and SPSS 17.0).

RESULTS

There was a distinct variation in wavelength of transmission spectra within each polyethylene shade, the actual spectral transmissions of 350 to 1000 nm for each of the colored polyethylene film are shown in Figure 1. As is expected, the control (non-shaded) had the highest level of illuminance followed by white; yellow and red, while the blue shaded plants received the lowest level of illuminance.

Biomass yield

The polyethylene shade used in this experiment manipulated the variety of lighting environment that influenced crops growth and yield of *C. indicum*. Biomass yields of different treatments as dry weight of one thousand buds or flower heads and the total number of buds and flower heads from individual plant were described in Table 2. Plants grown in open condition had highest mean weight of one thousand buds or flower heads at five harvesting stages (P<0.01). Herbs grown under blue polyethylene film had the lowest mean weight with significant statistical differences (P<0.01) with other treatments from visible immature buds to blossomy inflorescence matures. The weight of one thousand buds or flower heads gradually increased accompany with the increasing of harvesting stages in all cultivated conditions.

Table 2. Yields of buds or flowers of *Chrysanthemum indicum* L. grown under different colored plastic films and harvested from five flowering stages

Treatment	Weight (g) [a]					Total number [b]
	1	2	3	4	5	
Control	27.55±1.14dA	32.79±0.72cA	34.81±1.07cA	39.28±0.96bA	43.70±0.60aA	214±12E
White film	18.65±0.99dB	22.56±0.90cB	25.62±0.88bB	27.63±1.57bB	31.42±1.01aB	312±13C
Yellow film	12.37±1.31bC	12.86±1.73bC	14.88±0.96abC	16.31±1.03aC	16.77±1.06aC	437±23B
Red film	8.62±0.87cD	11.42±0.74bC	11.94±0.13bD	15.33±0.87aC	15.89±1.88aC	474±4A
Blue film	7.34±0.71cD	8.39±0.37bcD	10.70±1.06abD	11.15±1.06aD	11.81±1.35aD	269±12D

1, 2, 3, 4 and 5 express five flowering stages. [a] Weight was the dry weight of one thousand flower heads at five Stages. Data are expressed as the mean ± standard deviation and are the mean of three replicates. Means with small letter within the same row are significantly (P<0.01) different. Means with capital letter within the same column are significantly (P<0.01) different. [b] Total number of buds and flower heads from individual plant. Data are expressed as the mean ± standard deviation and are the mean of ten replicates. Means with capital letter within the same column are significantly (P<0.01) different.

The number of buds or flower heads of individual plant varies from 214±12 to 474±4 and the minimum is also obtained in herbs grown in open condition which is significantly lower (P<0.01) than that of those four shade treatments. Bud and flower head numbers of individual herb grown under red polyethylene film was substantially higher than other treatments (P<0.01). The number of buds and flower heads declined in the following order: red > yellow > white > blue > control.

Flavonoids

Figure 2a displays the impact of colored polyethylene and maturation on changes of total flavonoids. A significantly highest concentration of total flavonoids (49.04±2.00 mg/g, DW) in flowers grown under natural environment and harvested at Stage 3 was observed (P<0.01). Influences on contents of linarin are presented in Figure 2b. Flowers grown under blue film got the highest content (10.56±0.51 mg/g, DW) at Stage 3. The lowest concentrations of linarin, identified in flowers grown under white film at all five harvesting stages (P<0.01), were superior to those grown under other treatments. Effects on contents of quercetin are summarized in Figure 2c. The highest concentration of quercetin (4.00±0.02 mg/g, DW) was found at flowers grown under red film and harvested at Stage 3; Flowers grown under natural environment got the lowest content compared with those grown under other treatments at all five harvesting stages (P<0.01). Figure 2d shows the influence on contents of luteolin. Flowers grown under white film got the highest content (2.79±0.02 mg/g, DW) at Stage 3. Flowers grown under red film got the lowest content compared with those grown under other treatments at first four harvesting stages (P<0.01). Changes of apigenin affected by coloredpolyethylene and maturation are revealed in Figure 2e. Flowers grown under red film had a higher content compared with those grown under other treatments at first three harvesting stages (P<0.01), and got the highest content (3.48±0.04 mg/g, DW) at Stage 2. Flowers grown under white film got the lowest content compared with those grown under other treatments at all five different maturation stages (P<0.01). The highest acacetin content was determined at Stage 2 from red color polyethylene shade followed by control and yellow, while white polyethylene shade had the lowest content of acacetin among all shades at all five harvesting stages in Figure 2f.

Phenolic acids

Effects of colored polyethylene and maturation on development of chlorogenic acid are exhibited in Figure 2 g. Flowers grown under red film had a higher content compared with those grown under other treatments at first three harvesting stages (P<0.01), and got the highest content (2.32±0.03 mg/g, DW) at Stage 3. Flowers grown under yellow film got the lower content compared with those grown under other treatments at last four harvesting stages (P<0.01). Contents of caffeic acid affected by colored polyethylene and maturation are illustrated in Figure 2h.

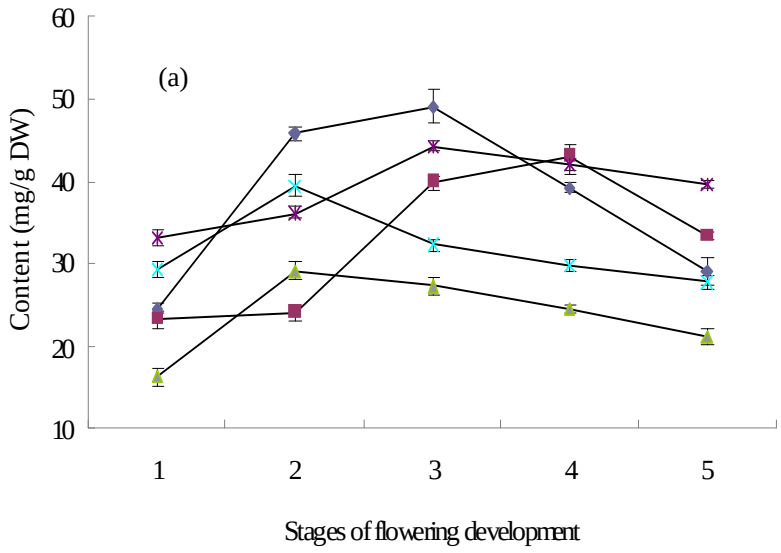

Figure 2a. Light quality effects on changes of flavonoids and phenolic acids levels in flowers of *Chrysanthemum indicum* L. during different maturation. (a), Total flavonoids.

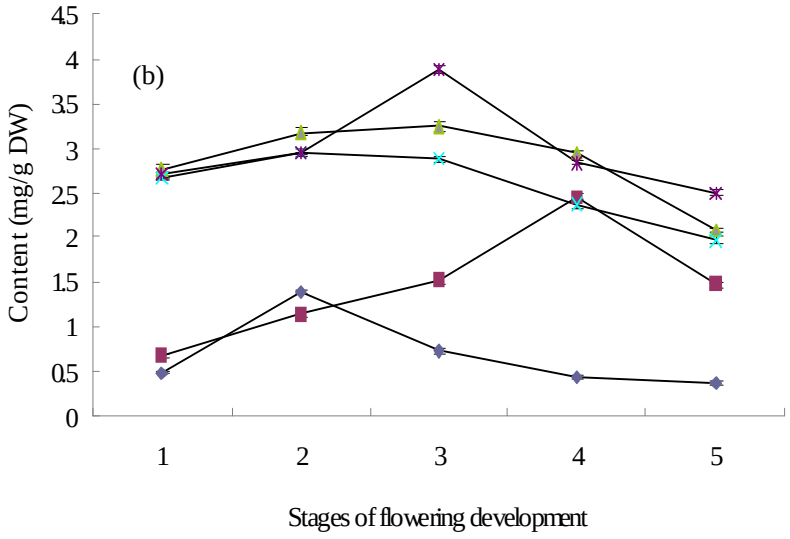

Figure 2b. Light quality effects on changes of flavonoids and phenolic acids levels in flowers of *Chrysanthemum indicum* L. during different maturation. (b), Linarin.

Flowers grown under white film had a higher content compared with those grown under other treatments at last three harvesting stages (P<0.01), got the highest content (98.40±0.61 µg/g, DW) at Stage 3. Flowers grown under red film got the lower content compared with those grown under other treatments at all five harvesting stages except Stage 2 (P<0.01).

DISCUSSION

Orzolek and Murphy (1993) and Decoteau et al. (1989) reported that polyethylene colors have distinct optical characteristics and thus reflect different radiation patterns into the canopy of a crop, thereby affecting plant growth and development. The different colored poly-ethylene

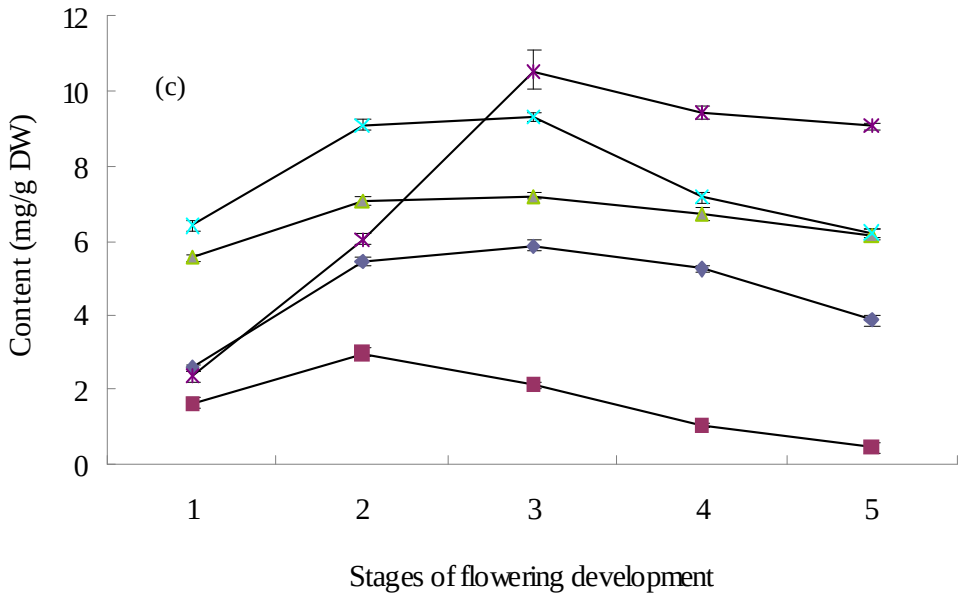

Figure 2c. Light quality effects on changes of flavonoids and phenolic acids levels in flowers of *Chrysanthemum indicum* L. during different maturation. (c), Quercetin.

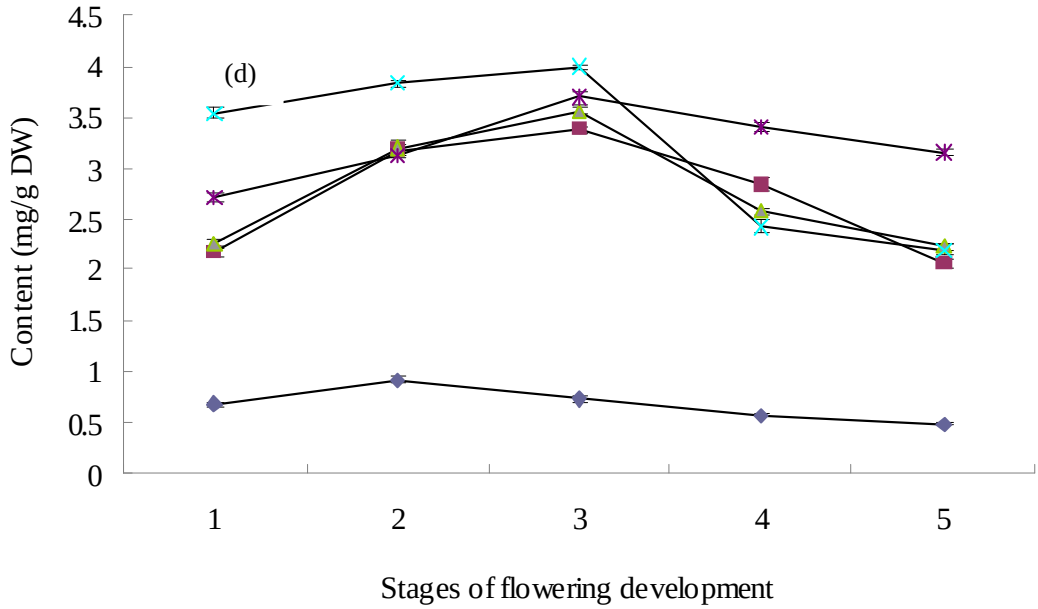

Figure 2d. Light quality effects on changes of flavonoids and phenolic acids levels in flowers of *Chrysanthemum indicum* L. during different maturation. (d), Luteolin.

was utilized as cover in this experiment, and they reflect different spectra of the visible light around *C. indicum* that may affect plant development and yield. The metabolites are unique to plants and are an essential part of their success in adapting to life in diverse and inconstant surroundings (Tian et al., 2008). It might putative that the treatments may have a profound influence on development of biomass yield and active components

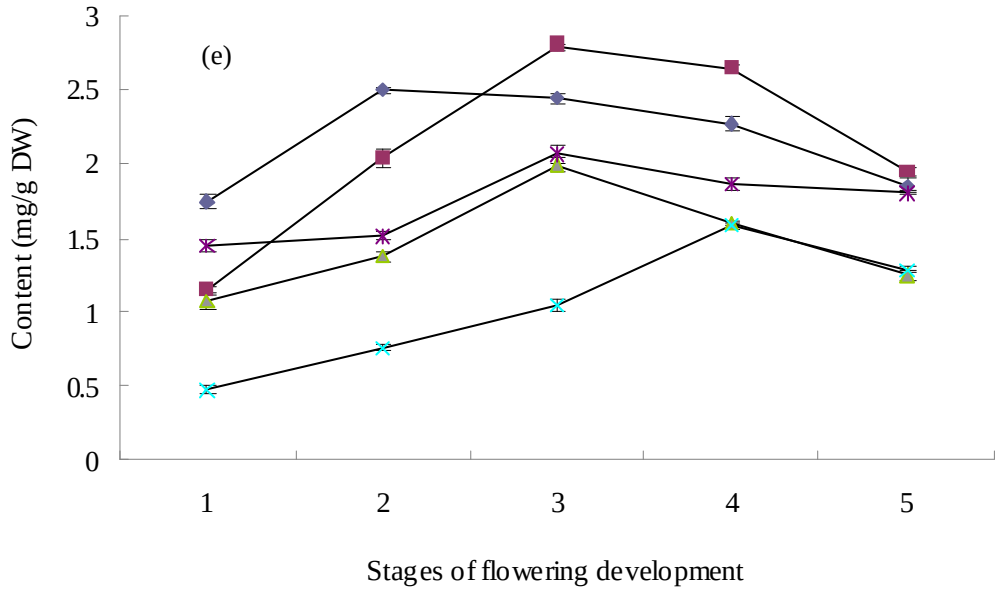

Figure 2e. Light quality effects on changes of flavonoids and phenolic acids levels in flowers of *Chrysanthemum indicum* L. during different maturation. (e), Apigenin.

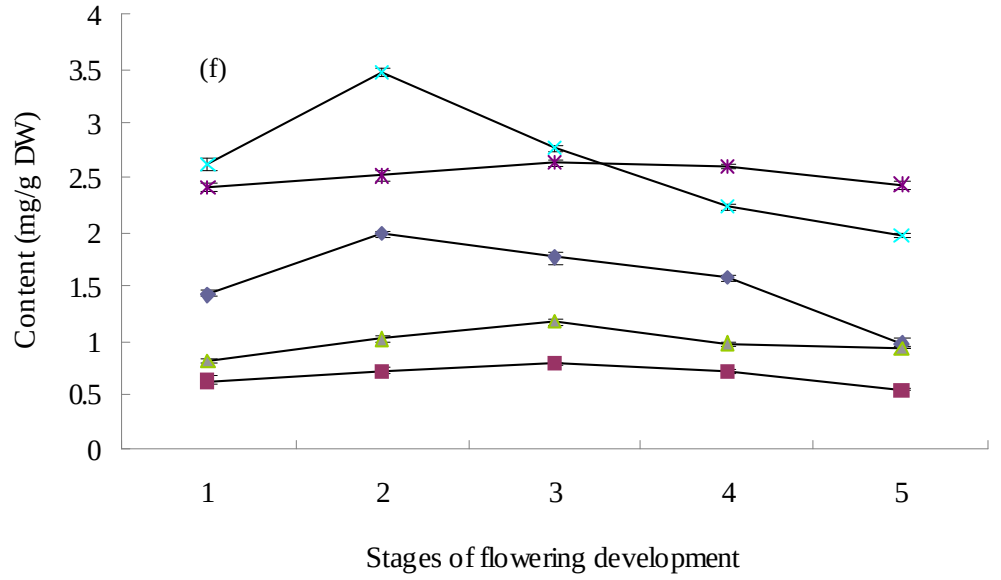

Figure 2f. Light quality effects on changes of flavonoids and phenolic acids levels in flowers of *Chrysanthemum indicum* L. during different maturation. (f), Acacetin.

in capitulum. In our manuscript, for yield evaluation, total number of flowers from individual plant and dry weight of one thousand buds or flower heads from different stages were determined; we also quantified the concentrations of some specific flavonoids and phenolic acids in the herb grown under different treatments. The study suggested that flavonoids and phenolic acids in flowers of *C. indicum* treated by different polyethylene colors and harvested at different flowering stages shared a similar qualitative composition, which differed mainly from the quantitative point of view. It was observed that the concentration of flavonoids and phenolic acids first increased as the

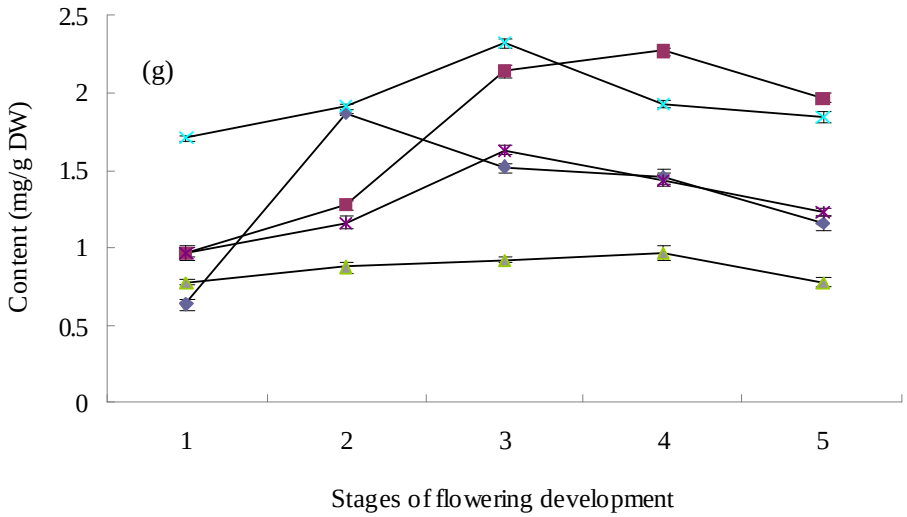

Figure 2g. Light quality effects on changes of flavonoids and phenolic acids levels in flowers of *Chrysanthemum indicum* L. during different maturation. (g), Chlorogenic acid.

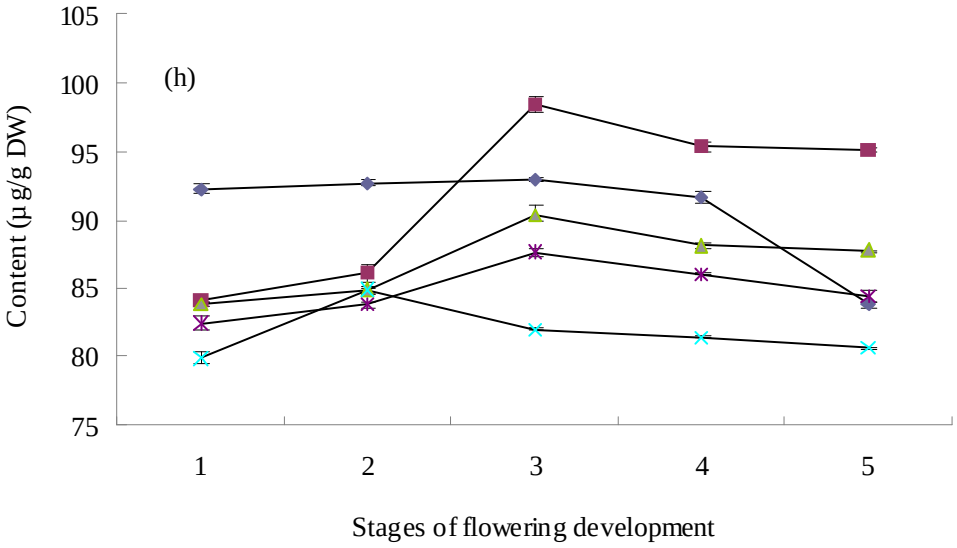

Figure 2h. Light quality effects on changes of flavonoids and phenolic acids levels in flowers of *Chrysanthemum indicum* L. during different maturation. (h), Caffeic acid. —◆—, Control; —■—, White film; —▲—, Yellow film; —✳—, Red film; —✱—, Blue film.

flower develops, but finally decreased. The results confirm that, the yield and phytochemicals of *C. indicum* was affected by different colored shade polyethylene and maturation. Effects of environmental factors on the formation of anthocyanin have been well documented (Awad et al., 2001), in the present experiments with flowers colored shade polyethylene treatments hardly affected the synthesis of flavonoids, accumulation of linarin, quercetin, apigenin, acacetin treated by blue and red film were recorded much higher than other treatments. Our data also conflict with the conclusion, the total flavonoids and luteolin are not enhanced by blue and red films. According to Casal (1994), some plants are more sensitive to blue light; Hemming et al. (2004) demonstrated that strawberry plants produce a higher yield, a higher number of fruits and a slightly higher mean fruit size under blue film. Similarly, *C. indicum* plants respond most to light in the blue, it was found that the blue

polyethylene shade condition produced more number of buds and flower heads and bioactive compounds total flavonoids, linarin, quercetin, apigenin, acacetin, chlorogenic acid and caffeic acid of *C. indicum*, compared with other conditions of films. Chlorogenic acid was apparently affected by red and white films; however, caffeic acid was affected by white film. Although the precise mechanism for the specific accumulation of chlorogenic acid and caffeic acid in capitulum remains unknown, it is possible that the formation activity of phenolic acids could, in part, be associated with light quality and/or maturation.

The results provided basic information for the development of the two main active components contents in flowers regulated by colored shade polyethylene treatments and harvesting stages. Based on the investigation, flowers with controllable content of phytochemicals could be easily obtained. Various environmental factors can affect plant growth and development. The contents of phytochemicals vary depending on climate, regions of cultivation and seasons of harvest (Zhang et al., 2007). Wang et al. (2009) reported that light is a predominant source of energy for plant photosynthesis and also an important signal for plant growth and development, It is known that not only are plants able to respond to the quality of light but also to its intensity and day length. In the present manuscript, though different polyethylene colors and maturation that showed high quantities of phytochemicals could be used, we hoped to identify additional environmental conditions, such as light quantity and photoperiod, which could be useful for improve phytochemical substances contents in the future.

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