Carotenoids of Durum Wheat: Induced High Pigment Levels Obtained by Treatment of the Growing Plant With Chlorophenylthiotriethylamine (CPTA) Hydrochloride [1]

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ABSTRACT

The effect of chlorophenythiotriethylamine (CPTA) on durum wheat cv. Hercules was examined. CPTA was applied by spraying at various stages of growth. Harvested grain showed a significant increase in pigment protein and steroid content. Optimal results were obtained with an application of 40 oz. per acre 1 week after flowering. Factors that would influence the interaction between seed protein, pigment, and steroid are discussed in relation to the statistical analysis of the levels of flour constituents. Methods of carotenoid and steroid analysis are discussed.

The carotenoid lutein is the major pigment in durum wheat and contributes to the desirable yellow color of its product. Durum wheat varieties with relatively high levels of this pigment have been developed by plant breeders. So far, the possibility

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of increasing pigment content of durum wheat by chemical treatment has not been investigated. Coggins et al. (1) showed that they could induce the formation of lycopene in grapefruits by treatment with CPTA. Lycopene is the carotenoid responsible for the red color in grapefruits and tomatoes. This suggested that treatment of durum wheat with CPTA might induce the formation of its native carotenoid, lutein.

This paper reports on the effect of CPTA treatment at different stages of development on a number of grain constituents of Hercules durum wheat. The main purpose of the study was to examine the effect of CPTA treatment on the yellow pigment content, since grain with a chemically induced high pigment level would provide excellent material for a comparative biochemical study of all aspects of grain-pigment biosynthesis.

MATERIALS AND METHODS

Hercules, a Canadian variety of durum wheat, was used for this study. The approved AACC method (2) for determination of yellow pigment in durum wheat has some inherent flaws; these were discussed by Lepage and Sims (3,4). If methanol is used as an extraction solvent instead of water-saturated *n*-butanol, optical density (OD) readings are higher and the ratio of solvent to sample weight is not as critical. With methanol as solvent, pigment determinations can be carried out on samples of 10 to 15 seeds, as well as on larger size samples. Accordingly, for this study methanol was chosen over water-saturated *n*-butanol as the extraction solvent in pigment determinations. Pigment values obtained by the AACC method, though not absolute, are well established and have the merit of relative proportionality. Accordingly, pigment values reported in this paper have been so calculated as to conform with values obtained by the AACC method. In the AACC method, pigment values are calculated by using an absorptivity of 1.6632; i.e., a solution of 1.0 mg. pigment in 100 ml. of solvent has an OD at 435.8 nm. of 1.6632 (2). To obtain pigment values that conform to standard method values, an absorptivity of Hercules extracts in methanol of 2.1249 was used. This absorptivity was calculated from the formula:

$$1.6632 \times \frac{\text{OD at 442 nm. (methanol extract 2 g. per 10 ml.)}}{\text{OD at 435.8 nm. (butanol extract 2 g. per 10 ml.)}}$$

For steroid analyses, a method used to determine serum cholesterol levels was adapted to determine β-sitosterol levels in grain. Protein levels were determined by the macro-Kjeldahl procedure.

The details of the procedures used for pigment and steroid analyses were as follows.

Pigment Analysis

Ten milliliters of methanol (purified over aluminum oxide) was added to 1.0 to 1.5 g. of flour or ground seed in a screw-capped vial. The contents were mixed by swirling and left overnight, mixed again in the morning, and left to settle. The supernatant was decanted into a cuvet and its spectrum was determined between 550 and 400 nm. on a Carey Spectrophotometer. OD at 442 nm. was used for calculation of pigment content. Pigment content was expressed in parts per million.

Steroid Analysis

Fifteen milliliters of a solution of 10% glacial acetic acid in chloroform was added to 2.0 to 3.0 g. of flour in a screw-capped vial. The contents were mixed by swirling and left overnight, remixed in the morning, and left to settle. Ten milliliters of the supernatant was transferred to another vial and evaporated to dryness. The residue was taken up in 1 ml. of glacial acetic acid, brought to the boiling point, and left to cool. Five milliliters color reagent was then added, and after 10 min. the OD of the resulting solution was read at 662 nm. The color reagent was prepared as follows: 1.45 g. *p*-toluene sulfonic acid was added to 600 ml. acetic anhydride and 400 ml. of glacial acetic acid. To 25 ml. of this solution, 5 ml. of concentrated sulfuric acid was added in portions with cooling. A sample of β-sitosterol was used to obtain a standard curve. Results are expressed in milligrams percent.

The grain that was analyzed was obtained from field experiments, so designed as to facilitate statistical analysis of the results.

Experiment I

This experiment was conducted during the summer of 1971. The experimental design was a four-replicate split plot, with plots consisting of nine rows, 9 ft. in length. CPTA was applied as an aqueous solution by means of a pressure sprayer calibrated to deliver 20 gal. per acre. Three rates of chemical, 8, 16, and 32 oz. per acre of active ingredient, were applied, at the three-leaf stage, at the boot stage, and 4 days after flowering, while the check plots received no treatment. Seed yield and 1,000-kernel weight were determined as well as total pigment content of Brabender-milled flour.

Experiment II

This experiment was conducted during the summer of 1972. Rates of CPTA application were 0, 24, 40, and 56 oz. per acre. Unsprayed check plots were also provided to test the actions of the wetting agent (Triton X-100) alone, if any. The spray was applied at two stages, 1 week and 2 weeks after flowering. The harvested grain was analyzed for total pigment of ground grain obtained from a Wiley mill.

Experiment III

This experiment was conducted during the summer of 1972. The experimental design was a four-replicate latin square. A new formulation of CPTA was used in the experiment, providing CPTA in the amine form. The chemical was applied one week after flowering at rates of 24, 40, and 56 oz. per acre of active ingredient. Check plots received no treatment. The harvested grain was separated into large and small seeds by sieving with a 5/64 X 3/4 in. sieve. Data obtained in this experiment included protein, pigment, and steroid content of Buhler-milled flour.

RESULTS

Results of each of the three field experiments will be presented separately.

Experiment I

Spraying with CPTA after flowering induced a significant increase in pigment content of the harvested seed (Table I). A rate of application of 32 oz. per acre after flowering gave a pigment content of 6.5 p.p.m. in the harvested seed, 20%

above pigment content of the control sample. The harvested grain was also analyzed for 1,000-kernel weight. Statistical analysis showed that this seed characteristic was not significantly affected by either the rate or the time at which CPTA was applied.

Experiment II

The results of this experiment were threefold. It showed that the wetting agent did not influence pigment content, that the time of spraying should be approximately 1 week after flowering for optimum effect of CPTA on pigment content, and that a rate of spray of approximately 56 oz. per acre should nearly maximize pigment increase (Table II). CPTA treatment at 56 oz. per acre increased seed pigment content as compared to lower treatment rates, but only when the treatment was applied 1 week after flowering.

Experiment III

Spraying with CPTA in the amine form did not result in larger pigment increases than those obtained in other experiments (Table III). Spraying with CPTA also positively affects both protein and steroid content. The effect predominates in the smaller size kernels; 40 oz. per acre gave the best results.

TABLE I. EFFECT OF CPTA ON MEAN PIGMENT CONTENT OF HERCULES DURUM WHEAT IN p.p.m. EXPERIMENT I[z]

Time of Spraying	No. Treat.	Rate of CPTA Spray, oz./acre			Average
		8	16	32	
Three-leaf stage	5.2a	5.2a	5.5ab	5.1a	5.25A
Boot stage	5.1a	5.1a	5.2a	5.4ab	5.20A
Post flowering	5.7ab	5.6ab	5.9bc	6.5c	5.93B

[z]Lower case letters, comparisons of four treatments at three stages of spraying with CPTA. Upper case letters, comparisons at three stages of spraying with CPTA. Means with like letters do not differ significantly at the 1% level by Duncan's multiple range test.

TABLE II. EFFECT OF CPTA ON MEAN PIGMENT CONTENT OF HERCULES DURUM WHEAT IN p.p.m. EXPERIMENT II[y]

Time of Spraying weeks after flowering	No. Treat.	Rate of CPTA Spray, oz./acre			
		0[z]	24	40	56
1	5.7a	5.7a	7.0b	7.1b	7.6c
2	6.0a	5.9a	6.3b	6.6c	6.6c

[y]Lower case letters, comparisons of five treatments within each stage of spraying with CPTA. Means with like letters do not differ significantly at the 1% level by Duncan's multiple range test.

[z]Water and wetting agent only.

Quality Analyses

The question remained whether this induced-high-pigment Hercules would compare favorably with the untreated Hercules in the macaroni-making process. Samples of each were therefore tested for their spaghetti-making quality [2]. The

[2]Courtesy Grain Research Laboratory, Winnipeg, Canada.

TABLE III. EFFECT OF CPTA ON MEAN PROTEIN, STEROID, AND PIGMENT CONTENT OF HERCULES DURUM WHEAT FLOUR. EXPERIMENT III[y]

Seed Size	Content	No. Treat.	Rate of CPTA Spray, oz./acre		
			24	40	56
Large	Steroid	99a	99a	104b	103a
	Protein[z]	15.0a	15.2a	15.2a	15.1a
	Pigment	5.3a	6.2b	6.5c	6.6c
Small	Steroid	100a	104a	109b	104a
	Protein	14.0a	14.4b	14.7c	14.6bc
	Pigment	6.3a	7.5b	7.7b	7.7b

[y]Lower case letters, comparisons of four treatments for each constituent within seed size. Means with like letters do not differ significantly at the 1% level by Duncan's multiple range test.

[z]Protein content in percent (N X 5.7 and 0% m.b.); pigment content in p.p.m., 0% m.b.; steroid content in mg. per 100 g., 0% m.b.

TABLE IV. CORRELATION COEFFICIENTS FOR THE DATA OBTAINED IN EXPERIMENT III

Class	Correlation Coefficient	Interaction		
		Pigment/steroid	Pigment/protein	Steroid/protein
Overall	Direct	+0.50**	−0.36**	−0.29**
	Partial	+0.44**	−0.25	−0.14
Large Seed size	Direct	+0.12	−0.07	+0.12
	Partial	+0.13	−0.08	+0.13
Small Seed size	Direct	0.43**	−0.15	−0.29
	Partial	+0.41**	−0.02	−0.26

**Significant at the 1% level, measured by the t test. n = 96 overall; n = 48 for seed sizes.

treated Hercules showed a higher pigment content as expected. Other test results were the same, except for protein content, which was shown to be 1.0% higher in the semolina of treated Hercules.

To test whether this difference in protein content was due to the action of CPTA, all samples in experiment III were analyzed for protein content and the data subjected to statistical analysis. CPTA significantly increased the protein content of Hercules durum wheat (Table III).

DISCUSSION

The observed pigment increases in treated crops showed the effect of CPTA on seed metabolism. The technique of using different chemicals to exert metabolic control is now widely used, e.g. weed control, membrane studies, and elucidation of biosynthetic pathways. It is not surprising, therefore, that a chemical would be found that exerts a promoter effect on carotenoid synthesis. Chemical modulating effects on carotenoid synthesis have been reported by Braithwaite and Goodwin (5), who showed that diphenylamine inhibits cyclization in carotenoids and Mummery and Valadon (6), who showed antimycin A can inhibit carotenoid synthesis.

In preliminary experiments, it seemed that CPTA showed a negative effect on normal carotenoid synthesis in durum wheat. When durum wheat was germinated in solutions of CPTA, it showed red coleoptiles. The red color was due to the formation of lycopene. This seemed like an inhibition of ring closure, so that no β-carotene could be formed. However, when the first leaf emerged, it showed only a red tip, while all subsequent leaves were normal in appearance. This could indicate that the action of CPTA was only of short duration, but further experiments showed a long-lasting effect of CPTA. If solutions of CPTA were injected in the stem after heading, the harvested seeds showed an increased pigment content. As a result of these preliminary findings, the field trials described in this paper were undertaken and a 20 to 30% increase in pigment level was obtained.

It is difficult to see by which mechanism CPTA could influence both protein and pigment. With regard to pigment, if CPTA affects the mevalonate pool, it should exert an influence on steroid as well as pigment as both share the same pathway up to farnasylpyrophosphate. The influences on protein might be secondary, through the formation of lipoprotein, as steroid and carotenoid are increased. A three-component multiple regression analysis might give an indication whether this is the case. To be able to do this multiple regression analysis, the samples in experiment III were analyzed for steroid content as well.

The results of this steroid analysis showed that CPTA treatment significantly increased the steroid level of the harvested seed. Where the pigment increase was 20 to 30%, the steroid increase was smaller, approximately 4%. If steroid and pigment show the same dependence on the mevalonate pool, they should show the same percentage increase. If their synthesis is compartmentalized, they should be independent of each other; e.g., C^{14} mevalonate fed to green seedlings does not appear in carotenoids of the chloroplast, since the chloroplast membrane is impermeable to mevalonate. In the endosperm, however, steroid and pigment might be expected to draw from the same mevalonic acid pool, but, if a cofactor like NADPH regulates the level of steroid synthesis independent of carotenogenesis, an increase in the mevalonate pool might be expected to increase the carotenoid level markedly, while only slightly increasing the steroid content.

The multiple regression analysis was carried out on the result of triplicate analyses for the three constituents on all samples of experiment III and showed, in the overall picture, significant correlation coefficients: positive between pigment and steroid, negative between protein and steroid, and negative between protein and pigment (Table IV). Considering the partial correlation coefficients, only at a constant protein value is there a significant interaction between pigment and steroid. Changes occur when the data are analyzed on the basis of two seed-size classes. No significant correlation coefficients were present in the analysis for large seeds while the picture for the small seeds was similar to the overall picture.

TABLE V. PROTEIN, STEROID, AND PIGMENT CONTENT OF HERCULES DURUM WHEAT FLOUR OF DIFFERENT SEED SIZES

Seed Size	Steroid mg. %	Protein %	Pigment p.p.m.	Steroid γ/seed	Protein mg./seed	Pigment γ/seed
Large	99	15.0	5.3	57	8.6	0.30
Small	100	14.0	6.3	41	5.7	0.26

That no significant correlation coefficients were observed in the analysis of large seeds must be due to the large amount of extra storage protein present in these seeds. This extra storage protein could mask any functional relationship that might exist between the three constituents. On a per-weight basis protein increased with seed size, whereas steroid and pigment declined. If these values were recalculated on a per-seed basis, the concentration of all three increased with seed size (Table V). Thus, there is a large amount of protein in the large seed that could not be related to pigment and steroid increases, and therefore gave rise to negative correlation coefficients between them in the overall picture. The positive correlation coefficient for steroid and pigment probably means that the action of CPTA is on an early precursor of carotenoid synthesis. These aspects need further investigation. We intend to use CPTA in a comparative study on the development of carotenoids in the durum wheat seed during the maturation process. Chen and Geddes (7) and Bouguerra (8) studied carotenoid development in one or more wheat varieties; a similar study on one variety with different pigment levels should enhance the value of these data.

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