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Is the Application of Carbendazim Harmful to Healthy Plants? Evidence of Weak Phytotoxicity in Tobacco

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To understand the phytotoxic effects that certain bezimidazole fungicides exert on plant growth, the aim of the present study was to determine the effect of the fungicide carbendazim, on foliar biomass, pigment content, and nutrient levels in tobacco plants (*Nicotiana tabacum* L. cv. Tennessee 86). The fungicide applied was carbendazim with a purity of 100%, at three different rates: 1.3 mM (carb1), 2.6 mM (the recommended concentration, carb2), and 5.2 mM (carb3). The control treatment was without carbendazim. The application of dosages of this fungicide lower than recommended (1.3 mM) resulted, on the one hand, in greater dry weight and, on the other, higher carotenoid concentrations, as well as higher N and K concentrations with respect to control. On the contrary, the application of the carbendazim dosage higher than recommended (5.2 mM) caused a decrease in dry weight and in all of the foliar pigments, as well as all of the nutrients, with respect to the other dosages and control. These results appear to indicate that besides its direct antibiotic action against pathogens, the effects of this fungicide in plants could be dangerous, especially at higher dosages. Nevertheless, the negative effects of carbendazim can be avoided by reducing the amount applied in current agriculture.

KEYWORDS: Carbendazim; phytotoxicity; *Nicotiana tabacum*

INTRODUCTION

In agriculture, plant diseases are controlled primarily by chemical products (fungicides, bactericides, nematicides, etc.). However, recent studies indicate that an average of 35% of possible agricultural yield is lost to fungal infections despite the extensive use of enormous quantities of fungicides (*1*). It is generally accepted that the activity of these compounds in the plant is derived from their antibiotic properties against the pathogen. However, few studies have addressed the question of whether these products alter or inhibit physiological and metabolic activities in the plant. In this respect, Bader and Abdel-Basset (*1*) showed for the first time that triforin fungicides (Saprol) strongly inhibit electron transport within the chloroplasts.

Benzimidazoles, a group of organic fungicides that act systemically, are widely used in agriculture. These compounds at relatively low concentrations control a broad range of fungi (*2*) and can provide certain physiological benefits for plants (*3*). For example, benomyl (carbamate of methyl-*N*-(butylcarbamyl)-benzimidazole), one of the most effective of the benzimidazoles and extensively used, has a cytokine-type activity in soy and radish (*4*). Meanwhile, carbendazim (benzimidazol-2-yl methyl carbamate), with properties similar to those of benomyl and of which it is an active metabolite, retards senescence in wheat (*5*).

Nevertheless, some benzimidazoles can be phytotoxic. For example, benomyl reduced the growth of cucumber (*6*), lettuce (*7*), and loblolly pine and other species (*8*). In addition, benomyl reportedly caused vein discoloration in Swedish ivy (*9*), whereas tomato seedlings became chlorotic and stunted at higher rates (*10*). These results demand further research into the phytotoxic effects of benzimidazole fungicides, as it remains unclear which physiological processes are affected.

Given that leaves, stems, and roots respond to environmental stimuli (as in the case of pesticide application), it would be expected that metabolic responses to environmental stress largely regulate plant growth and development. It is worth emphasizing the effect of the nutritional state, because many studies have shown that nutrients (primarily N, P, and K) can determine the production and distribution of dry matter in the different parts of the plant (*11*, *12*). In tobacco plants, Balachandran et al. (*13*) showed that changes in the nutritional state of these essential macronutrients could alter these parameters.

The fungicide used was carbendazim (carb) because (1) it is one of the most widely used fungicides in southeastern Spain, a zone of intensive agriculture, and (2) because, being a fungicide of broad preventive spectrum, it is applied to a large proportion of the crop that is not even infected by the pathogen (*14*). To understand the effects of fungicides on plants, we analyzed the way in which different concentrations of the

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fungicide carbendazim influence the biomass production, pigments content, and nutrient levels in tobacco plants. The plants chosen for this experiment were tobacco. Prior research on this plant in our laboratory has revealed a substantial influence of carbendazim in the metabolism of phenolic and oxidative compounds, which are known to have a key role in plant defensive responses as well as in plant development under diverse conditions of stress (*15*, *16*).

MATERIALS AND METHODS

Crop Design and Plant Sampling. Seeds of *Nicotiana tabacum* L. cv. Tennessee 86 were sown in May (1999) in southern Spain (Granada). The seedlings were grown in individual pots of peat in an experimental greenhouse for 45 days and then were transferred to individual pots (25 cm upper diameter, 17 cm lower diameter, 25 cm in height), filled with vermiculite. The plants were grown in a cultivation chamber under controlled environmental conditions with relative humidity of 60−80%, temperature of 30/20 °C (day/night), and a 16-h photoperiod at a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} (measured at the top of the plants with a 190 SB quantum sensor, Li-cor Inc., Lincoln, NE). For 1 month (from day 45 until day 75 after sowing), before the experimental treatments, the plants received a nutrient solution of 6 mM KNO_3, 2 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 1.5 mM $MgSO_4$, 5 μM Fe-EDTA, 2 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, and 2.5 μM H_3BO_3. The nutrient solution (pH 5.5−6.0) was renewed every 3 days.

At 75 days after sowing, the treatments were applied to plants as aqueous foliar sprays containing the surfactant Tween 20 (0.5% v/v), using a stainless steel sprayer. The fungicide applied was carb (benzimidazol-2-yl methyl carbamate; $C_9H_9N_3O_2$) with a purity of 100% at three different rates: 1.3 mM (carb1), 2.6 mM (the foliar application rate recommended by the manufacturer, carb2), and 5.2 mM (carb3). The control treatment received no carbendazim application.

The experimental design was a randomized complete block with four treatments, arranged in individual pots with six plants per treatment, each replicated three times. Each treatment was applied three times every 2 weeks. The plants were sampled just prior to the onset of flowering. From each plant, leaves were sampled only once, on day 135 after sowing. All of the leaves sampled were in the mature state, with lengths of >10 cm. The leaves were rinsed three times in distilled H_2O after they had been disinfected with nonionic detergent at 1% (v/v) (Decon 90, Merck) (*17*) and then blotted on filter paper. A subsample of leaves was used fresh for the analysis of the pigment content (triplicate assays were performed for each extraction), whereas the other subsample was dried in a forced air oven at 70 °C for 24 h and then used for the analysis of the nutrient concentrations. Dry weight was recorded and expressed as grams of dry weight (DW) per leaf.

Plant Analysis. *Determination of Pigments.* To measure chlorophylls and carotenoids, we followed the procedure described by Wellburn (*18*). To determinate the foliar area, leaf disks of 7 mm diameter and ~0.125 g were collected. The chlorophyll (*a* plus *b*) and carotenoid contents were expressed as micrograms of chlorophyll per square centimeter, using Wellburn equations (*18*).

Determination of Total Nutrients. A 0.1 g DW subsample was digested with sulfuric acid and H_2O_2 (*17*). Total nutrients were determinated after dilution with Millipore water.

Total N was measured as detailed for the NH_4^+ procedure (*19*). The results were expressed as milligrams of organic N per gram of DW.

Total P was analyzed according to the vanadomolybdophosphoric colorimetric method at 430 nm (*20*) using dry mass digested with 12 N H_2SO_4 and 30% H_2O_2. Results of P were expressed as milligrams per gram of DW.

The total cation content, Ca and Mg, was analyzed by atomic absorption spectrophotometry as described by Hocking and Pate (*21*), and K was determined following the procedure of Lachica et al. (*22*). All cations were expressed as milligrams per grams of DW.

Statistical Analysis. Standard analysis of variance was used to assess the significance of the treatment means. The data shown are presented as mean values ± standard error (SE). Differences between treatment means were compared using the LSD at the 0.05 probability level. Also, a correlation analysis was made between the different variables. Levels of significance were represented by * at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, and NS (not significant).

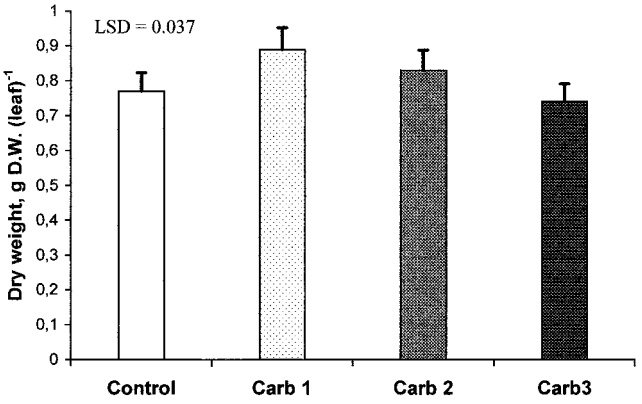

Figure 1. Effects of the application of carbendazim on dry weight in tobacco leaves. Data are means ± SE ($n = 3$).

RESULTS AND DISCUSSION

Biomass Production. Except for carb3, the other treatments with carbendazim significantly increased dry weight with respect to control ($P < 0.001$; Figure 1). Nevertheless, the greater amounts of carbendazim applied resulted in a gradual decline in biomass production, the lowest values appearing in the carb3 treatment, which significantly diminished dry weight by 17% with respect to control. Thus, according to our results, the application of carbendazim in tobacco plants can be beneficial, augmenting dry weight at the recommended dosage of 2.6 mM (carb2) and especially at the lower concentration of 1.3 mM (carb1). Tripathi et al. (*5*) observed that the application of carbendazim at a concentration of 20 μg mL^{-1} inhibits the breakdown of chlorophyll as well as RNA and proteins, because it protects the leaves, preventing the flow of ions responsible for the degradation of cell membranes. This could explain the beneficial effect of carbendazim on foliar biomass production found in our experiment, especially when the fungicide was applied in optimal concentrations. On the other hand, the reduced dry weight in the treatment of 5.2 mM (Carb 3), exceeding recommended levels, implies that excess amounts of this fungicide can harm the normal development of healthy tobacco plants. In fact, these plants were characterized by such toxicity symptoms as chlorotic burn and necrotic patches on the margins and tips of older leaves. Tripathi et al. (*5*) also demonstrated that the application of carbendazim at a concentration of 100 μg mL^{-1} increased the efflux of ions and amino acids, suggesting a disorganization of the plasma membrane, a fact that might explain the reduced foliar biomass observed in the present work after the application of the highest carbendazim dosage, carb3 (5.2 mM). The same researchers found that such treatment with carbendazim was phytotoxic and caused chlorosis and necrosis in leaves. These findings appear to explain the harmful effects caused by the fungicide in our experiment.

Pigments. Foliar pigments have been used in different plants, including tobacco, as indicators of biomass production and nutritional status, mainly for NPK. In our experiment, all of the carbendazim treatments lowered, but not significantly, chlorophyll concentrations (chl *a* and *b*), with respect to control values (Table 1). In addition, the content of these pigments gradually declined with greater carbendazim dosages, the lowest pigment values appearing with the strongest carbendazim application (carb3), some significantly 20% lower than control.

Table 1. Effects of the Aplication of Carbendazim on Pigment Concentrations in Tobacco Leaves [Data Are Mean ± SE ($n = 3$)]

treatment	chl *a* (μg cm^{-2})	chl *b* (μg cm^{-2})	carotenoids (μg cm^{-2})
T0	67.18 ± 0.41	26.52 ± 0.25	3.72 ± 0.08
carb1	66.82 ± 0.39	25.88 ± 0.23	4.8 ± 0.09
carb2	62.46 ± 0.36	24.98 ± 0.23	4.64 ± 0.09
carb3	54.14 ± 0.31	21.9 ± 0.19	3.9 ± 0.06
LSD	1.88	1.15	0.13
significance	$P < 0.01$	$P < 0.05$	$P < 0.001$

Similarly, Mihuta-Grimm et al. (*10*) noted chlorosis and stunting in tomato with the application of high rates of benomyl. Finally, the correlations found between dry weight and the concentrations of foliar pigments were positive and significant (dry weight of chl *a*, $r = 0.64$*; dry weight of chl *b*, $r = 0.58$*). Mackown and Sutton (*23*) demonstrated that the chlorophyll concentration in tobacco leaves is strongly related to yield. Therefore, the gradual decline in dry weight in the carbendazim treatments, and especially carb3, can be attributed in part to the lower chlorophyll concentrations caused by these treatments.

Meanwhile, the behavior of carotenoids, after the various carbendazim treatments, followed a pattern opposite that of the chlorophylls, because carotenoids content significantly increased in all treatments with respect to control (Table 1). However, as with the chlorophylls, the higher dosages of carbendazim gradually diminished carotenoid levels, the highest treatment, carb3, resulting in the lowest levels of these pigments, some 20% lower than for carb1. In addition, we found a close relationship between the carotenoid content and biomass production (dry weight of carotenoids, $r = 0.92$***). Carotenoid synthesis proved to be affected not only by irradiance itself but also by ambient temperature, nutrient availability, and other factors that can constrain the photosynthetic utilization of light (*24*). In this way, and especially under certain types of environmental stress, plants with low nutrient availability should exhibit larger xanthophyll cycle pools than plants with high nutrient availability (*25*). In this case, our results appear to indicate that the treatments with carbendazim can trigger a photoprotection mechanism in the plants via the carotenoids, which would interact with the light to alleviate the damage caused by the fungicide application (*26*, *27*).

Macronutrients. The concentration of N was not significantly altered by the lower carbendazim dosages (1.8 and 2.6 mM), whereas higher dosages (5.2 mM) caused a significant 6% reduction with respect to control (Table 2). On the other hand, the P concentration was diminished but not significantly, by all of the treatments (Table 2), the least effect being exerted by carb3, with a significant reduction of 20% with respect to control. Finally, all of the carbendazim treatments increased K concentrations significantly (Table 2) with respect to control; the highest concentration of this element registered for carb1.

Numerous studies have shown close positive relationships in tobacco plants between yield and N fertilizer as well as between yield and foliar N concentrations (*28*, *29*). In this way, our data indicate that the gradual decline in dry weight with increasing dosages of carbendazim may be due largely to the reduction in N accumulation, given the positive and significant correlation found between the two parameters (dry weight of N concentrations, $r = 0.76$**). Also, we found a positive and significant correlation between N and foliar pigments (chl *a* N concentrations, $r = 0.88$***; chl *b* N concentrations, $r = 0.93$***, carotenoid N concentrations, $r = 0.63$*). Previously, Mackown and Sutton (*23*) demonstrated that the chlorophyll concentration in tobacco leaves is firmly related to the foliar N concentration and therefore with yield.

Phosphorus is an essential element for higher plants and required in substantial concentrations in plant tissues, being particularly critical during vegetative growth. In terms of dry matter yield, the roots are much less affected by P deficiency than are the leaves (*30*). Nevertheless, in our experiment, the correlation found between P levels and foliar biomass was not significant (dry weight of P concentrations, $r = 0.14$ NS).

Another essential macronutrient for normal plant growth is potassium (K). This nutrient plays an important role in plant growth and metabolism (*31*). This fact could explain the positive and significant relationship found in our experiment between dry weight and K concentrations ($r = 0.66$*). Our results support numerous works that show how K and N are strongly related to yield in most crops (*32*−*34*).

Thus, we find that N, P, and K were similarly affected by the carbendazim application, their concentrations decreasing with greater amounts of the fungicide. That is, the strongest decrease corresponded consistently to carb3 (5.2 mM), the carbendazim dosage higher than recommended. This reflects the fact that excessive application of this fungicide clearly hampers the accumulation of these elements in foliar tissues, either by inhibition of their absorption in the roots or by their translocation to the aerial part. In addition, it is noteworthy that the carb3 treatment had the largest reduction in dry weight with respect to control (Figure 1). Meanwhile, the application at lower than the recommended rate resulted in a higher concentration of these elements with respect to the other dosages applied (Table 2) and gave the highest dry weight values (Figure 1).

Cations such as K, Ca and Mg are important major nutrients for higher plants. Deficiencies in K and Mg result in an accumulation of photosynthates in the leaf, diminishing their distribution to dependent organs such as fruits (*12*). In addition, deficiency in these cations causes an accumulation of oxide species and afterward foliar chlorosis (*35*). In terms of Ca and Mg, the carbendazim treatments brought about comparable effects, significantly reducing the concentrations of the two elements with respect to control (Table 2). In addition, the application of increasing amounts of the fungicide progressively diminished the Ca and Mg levels, carb3 having the lowest levels of these elements (9 and 17%, respectively, lower than control). Marc et al. (*36*) reported similar results and also noted reduced foliar Ca levels with carbendazim application.

Table 2. Effects of the Aplication of Carbendazim on Macronutrient Contents in Tobacco Leaves [Data Are Means ± SE ($n = 3$)]

treatment	N (mg/g of DW)	P (mg/g of DW)	K (mg/g of DW)	Ca (mg/g of DW)	Mg (mg/g of DW)
T0	41.3 ± 0.35	10.76 ± 0.16	32.5 ± 0.29	11.1 ± 0.09	7.8 ± 0.07
carb1	41.7 ± 0.37	9.73 ± 0.15	36.7 ± 0.32	10.5 ± 0.06	6.4 ± 0.06
carb2	41.6 ± 0.37	8.61 ± 0.09	35.4 ± 0.29	10.2 ± 0.06	6.7 ± 0.04
carb3	38.9 ± 0.31	8.47 ± 0.09	34.1 ± 0.23	10.1 ± 0.05	6.5 ± 0.04
LSD	1.82	0.32	1.24	0.37	0.11
significance	$P < 0.05$	$P < 0.01$	$P < 0.001$	$P < 0.05$	$P < 0.001$

Taking into account all of the parameters analyzed, we found that in relation to the control, the different carbendazim treatments generally produced the same effects. The application of lower than recommended dosages of this fungicide (1.3 mM) resulted in greater dry weight as well as higher carotenoid concentrations and higher N and K concentrations with respect to the control. This reflects the beneficial effect that the foliar application of carbendazim can exert on the accumulation of these essential nutrients as well as on growth and development in tobacco plants.

The application of the recommended dosage of carbendazim (2.6 mM) slightly diminished dry weight and nutrients levels, as well as all of the foliar pigments, with respect to the lower dosage and in the case of chl (*a* plus *b*) as well as P, Ca, and Mg with respect to control values, too. These results show a harmful effect by carbendazim, although not severe, on nutrient status, foliar pigment synthesis, and biomass production. These results only partially support the recommendation of the use of this product because it represents slight phytotoxicity.

Finally, we found that the application of the carbendazim dosage higher than recommended (5.2 mM) caused the strongest decrease in dry weight and in all of the foliar pigments, as well as all of the nutrients with respect to the other dosages and control. These data clearly demonstrate that this fungicide, applied in excess, is phytotoxic for healthy tobacco plants. In addition, these results may to a large extent explain the necrosis shown by old leaves.

In short, our experimental results appear to indicate that the negative effects of carbendazim can be avoided by reducing the amount applied in current agriculture. On the other hand, there is a possibility this reduction in the fungicide could prove to be less effective in combating pathogens, but this possibility requires further investigation in infected plants.

ABBREVIATIONS USED

Carb, carbendazim; PPFD, photosynthetic photon flux density; chl, chlorophyll.

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