# Flavonoid Phytoalexins Are Synthesized by Diverse *Beta vulgaris*

**Susan S. Martin**
*USDA-ARS, Crops Research Laboratory,*
*1701 Center Ave., Fort Collins CO 80526*

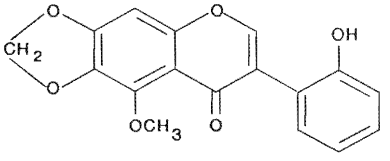
## ABSTRACT

**The ability of diverse *Beta vulgaris* to synthesize the flavonoid phytoalexins betagarin and betavulgarin was examined in 35 lines, including such diverse phenotypes as sugarbeet, table beet, fodder beet, and chard. Plants were field-inoculated with *Cercospora beticola* to produce Cercospora leaf spot disease. Extracts of necrotic lesions from leaves of every line contained the isoflavone phytoalexin betagarin, whereas the flavanone betavulgarin sometimes was not detected. The genetic capacity to activate the multiple biochemical pathways leading to accumulation of betavulgarin may be presumed to require constant selection, and the maintenance of this capacity in such genotypically varied lines is consistent with the presumed role of betavulgarin in resistance to Cercospora leaf spot.**

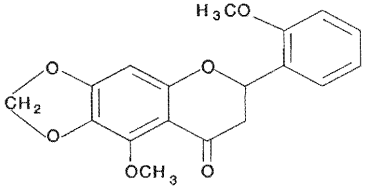
**Additional Key Words:** Sugarbeet; *Cercospora beticola*; flavonoid; isoflavone; flavanone; betagarin; betavulgarin; 2'-hydroxy-5-methoxy-6,7-methylenedioxyisoflavone; 5,2'-dimethoxy-6,7-methylenedioxyflavanone; Cercospora leaf spot; host-pathogen coevolution; HPLC.

Phytoalexins are low molecular weight, antimicrobial compounds that are synthesized by and accumulated in plants after exposure to microorganisms (Paxton, 1980, 1981). The ability to biosynthesize toxic chemicals that are not present prior to infection provides plants with an inducible biochemical defense mechanism against establishment or extensive development of a pathogen (Harborne and Ingham, 1978). Two such compounds, betavulgarin (abbreviated BV; 2'-hydroxy-5-methoxy-6,7-methylenedioxyisoflavone) and betagarin (B; 5, 2'-dimethoxy-6,7-methylenedioxyflavanone) (Fig. 1) are accumulated by sugarbeet, *Beta vulgaris* L., in response to infection by the fungus *Cercospora beticola* Sacc. (Geigert et al., 1973). The accumulation of these flavonoid compounds in Cercospora leaf spot (CLS) diseased plants has been studied in several commercial sugarbeet cultivars, as well as in sugarbeet lines selected for resistance to CLS (Johnson et al., 1976; Martin, 1977; Martin, unpublished). However, no studies have explored the ability of genotypically diverse *Beta vulgaris* lines to synthesize and accumulate these phytoalexins under CLS infection.

**Figure 1.** Structures of betagarin and betavulgarin.




BETAVULGARIN
2'-hydroxy-5-methoxy-6,7-methylenedioxyisoflavone




BETAGARIN
5,2'-dimethoxy-6,7-methylenedioxyflavanone

The species *Beta vulgaris* in the broad sense includes a group of diverse phenotypes that are believed to have been derived by human selection from a common progenitor. Sugarbeet, fodder beet, table beet, and chard are among the phenotypic extremes achieved through this relatively recent selection process. The

steps in the domestication and diversification of beets are unknown, but it is thought that the ancestral beet type may have been the sea-beet, *Beta maritima* L. [syn. *Beta vulgaris* L. var. *maritima* Moq.; *B. vulgaris* ssp. *maritima* (L.) Arcang.; *B. vulgaris* L. var. *maritima* (L.) Boiss.], a halophytic plant found in the Mediterranean, Asia Minor, Asiatic steppes, eastern India, and westward to the Canary Islands and along the Atlantic Coast of Europe to the North Sea (Coons, 1954). It is reasonable to assume that the Mediterranean ancestors of the cultivated beet types had a close coevolutionary association with *Cercospora beticola,* which is ubiquitous today throughout that region. Coons (1975) commented that in 1925, when he examined the *Beta maritima* specimens at herbaria in England and France, "The herbarium plants were almost universally affected by Cercospora leaf spot." If B or BV is involved in resistance to Cercospora leaf spot disease, which is present everywhere beets are grown, then the phytoalexins should be maintained through cultivar development; i.e., a successful beet population, whether native or cultivar, must have at least some resistance to the disease. By this reasoning, the capacity to synthesize and accumulate B or BV should be found in every derived cultivar or line of *Beta vulgaris.* The objective of the study reported here was to determine the generality of phytoalexin production in Cercospora leaf spot diseased plants of a diverse group of native and cultivated forms of *Beta vulgaris.* An on-going Cercospora leaf spot disease evaluation at Ft. Collins of *Beta* lines from the germplasm collection of the USDA-ARS North Central Plant Introduction Station at Ames, Iowa, provided a fortuitous opportunity for such a study.

## MATERIALS AND METHODS

Because the concept of this work was to determine whether the capacity for flavonoid phytoalexin production was absent in any *Beta vulgaris* line that could be tested, samples were obtained at various times and under various circumstances. In every case, however, the sampled plants were field-grown in the USDA-ARS Cercospora leaf spot test nursery at Fort Collins, CO, in 1987, 1988, or 1989. Plants were grown and inoculated with *Cercospora beticola* spore suspension as described previously (Ruppel and Gaskill, 1971). After inoculation, plants were watered frequently by means of overhead sprinklers to promote development of Cercospora leaf spot. The necrotic leaf lesions characteristic of the disease were sampled with a hand-operated punch. Both the necrotic zone and a ring of surrounding green tissue were included in each tissue disk ("lesion") obtained with a 4.8 mm (inside diameter) punch. Each sample consisted of ten punched lesions, composited from

at least five plants. Within 1 h of collection, samples were extracted by sonicating for 15 m with diethyl ether to cover. The extracted lesions were removed, and the extract was evaporated to dryness at 23C under a gentle stream of nitrogen. To each sample was added 0.200 mL of a 1:1 v/v mixture of acetonitrile (HPLC-grade) and 3% (aq.) acetic acid. Each sample was filtered through a 0.2 μ syringe filter prior to analysis by HPLC (Martin, 1989). B and BV were identified by their UV spectra, which were obtained on-line from a photodiode array detector and recorded. The chromatogram of each sample was recorded at 280 nm and peak areas were determined with an electronic integrator; samples were analyzed at a detector sensitivity of 0.1 absorbance unit full scale (AUFS), and an integrator attenuation of X 512.

## RESULTS AND DISCUSSION

Table 1 summarizes the lines or PIs sampled, and the presence or absence of B or BV in the test sample. Many lines were sampled several times, but only summary results are listed in Table 1. Quantitative data were obtained for B and BV in each sample, but because the purpose of this investigation was simply to examine the presence or absence of B and BV in thediverse lines, no concentration data are presented. This decision was made largely to avoid the temptation to make quantitative comparisons among lines, which is not justified because not all samples were obtained under identical circumstances. B and BV concentrations change significantly over the first several days that lesions are visible (Martin, unpublished), and environmental conditions, particularly temperature and humidity, affect lesion development; thus, it is not appropriate to make quantitative comparison of lesions of different ages, collected in different years, as were the samples of this study.

Betavulgarin was detected in every sample from every *Beta* line tested (Table 1). Betagarin, however, was not present at detectable levels in some samples. Both compounds arise *via* a common biosynthetic pathway, in which flavanones are biosynthetic precursors of isoflavones (Hahlbrock and Grisebach, 1975; Hagmann and Grisebach, 1984; Stafford, 1990). B and BV do not have identical substituents, so a direct conversion of the flavanone B to the isoflavone BV cannnot be postulated, but the lack of significant B in some samples may simply indicate that it has been transformed to other products in the biosynthetic sequence. In some samples there occur, usually in small quantities, three other compounds whose ultraviolet spectra and HPLC retention behavior suggest that they are flavanones closely related to betagarin (Martin, unpublished). It seems likely that these are the three flavanones that, together

**Table 1.** *Beta vulgaris* lines, infected by *Cercospora beticola*, tested for accumulation of the phytoalexins betagarin (B) and betavulgarin (BV). Lines with PI numbers are from the USDA-ARS Plant Introduction Station, Iowa State Univ., Ames, IA. [+ = Compound present; tr = trace amount detected; nd = not detected; LSR = leaf spot resistant; LSS = leaf spot susceptible]

| ID Nr | Country of Origin | Line or PI Number | Description or comment | Presence of BV | B |
|---|---|---|---|---|---|
| 1 | U.S. | — | Long-term LSR check (Ft. Collins) | + | tr |
| 2 | U.S. | — | Long-term LSS check (Ft. Collins) | + | + |
| 3 | Turkey | PI-120693 | Green leaves; yellow and orange roots | + | + |
| 4 | India | PI-164805 | 'Choghundar'; table type; red leaves, roots | + | + |
| 5 | India | PI-164806 | 'Palak'; fibrous rooted, procumbent annual | + | + |
| 6 | Yugoslavia | PI-357351 | 'Polsko'; table type; red leaves, roots | + | + |
| 7 | Yugoslavia | PI-357360 | 'Ohridska Zolta'; Fodder type | + | + |
| 8 | Australia | PI-289692 | 'Fordhook Master'; Chard | + | tr |
| 9 | Yugoslavia | PI-357359 | 'Domasne'; Large, erect annual | + | nd |
| 10 | Poland | PI-285592 | 'Crassa Strzelecki I Har'; fodder type; yellow roots | + | nd |
| 11 | Poland | PI-285594 | 'Crassa Walcowaty Zolty Gr'; green leaves with yellow petioles; orange and yellow roots | + | + |
| 12 | Iran | PI-140351 | Erect or procumbent; white or pink roots | + | + |
| 13 | USSR | PI-355958 | 'V 19'; Sugarbeet type | + | + |
| 14 | Gr. Britain | PI-323938 | 'Avon Early'; Red table-beet type | + | + |
| 15 | U.S. | FC-607 CMS | Ft. Collins inbred; high LSR | + | + |
| 16 | U.S. | FC-607 | " " " | + | + |
| 17 | U.S. | SP 85700-0 | Smooth root (sugar x table) | + | + |
| 18 | Japan | — | 'Monohikari' (commercial sugarbeet) | + | + |
| 19 | U.S. | — | Yellow leaf mutant (Ft. Collins 821052) | + | + |
| 20 | China | CH-7805 | Sugarbeet | + | + |
| 21 | China | CH-Tianyan | Sugarbeet | + | + |
| 22 | China | PI-467875 | 'Shuang feng 6'; sugarbeet | + | + |

| ID Nr | Country of Origin | Line or PI Number | Description or comment | Presence of BV | B |
|---|---|---|---|---|---|
| 23 | China | PI-467878 | 'Shuang feng 304'; sugarbeet | + | + |
| 24 | Iran | PI-142820 | 'Choghondar' | + | + |
| 25 | Poland | PI-286501 | 'Poly Mono Ihar' | + | + |
| 26 | China | PI-105335 | 'Tzu Lo Pu Tou'; most roots red | + | tr |
| 27 | Turkey | PI-120706 | Some roots red; some bolters | + | tr |
| 28 | Manchuria | PI-141919 | Most roots red | + | + |
| 29 | Iran | PI-142814 | 'Choghondar' | + | + |
| 30 | Iran | PI-142816 | 'Choghondar'; some roots red; bolter | + | nd |
| 31 | Iran | PI-148625 | 'Chaghonda'; most roots red | + | tr |
| 32 | Turkey | PI-164978 | 'Cicla'; 'Pazi'; some roots red; annual | + | tr |
| 33 | Turkey | PI-171518 | | + | tr |
| 34 | U.S. | FC502-3 CMS X FC607 | Hybrid; LSR | + | + |
| 35 | U.S. | SP8540-0 | *Sclerotium rolfsii* resistant inbred | + | + |

with betagarin, represent all possible combinations of methoxy or hydroxy substitution at the 5 and 2′ positions (Fig. 1; i.e., 5,2′-dihydroxy; 5-hydroxy-2′-methoxy; 2′-hydroxy-5-methoxy; and 5,2′-dimethoxy). Thus, small amounts of the flavanone equivalent of betagarin may be detectable on occasion, although normally it is converted to the isoflavone. Structural characterization of the three unknown flavanones is in progress.

Isoflavonoids such as BV are well-known as phytoalexins, although they are produced most commonly by members of the plant family Fabaceae (Leguminosae) (Ingham, 1982, 1983). However, among the hundreds of known isoflavonoids, betavulgarin thus far is unique to members of the genus *Beta*, as it has not been reported from any other plant taxon, even its botanical relative, spinach *(Spinacia)*, a member of the same plant family (Richardson, 1981). BV, in particular, is effective in limiting the growth of *C. beticola* in *in vitro* bioassays (Johnson et al., 1976; Martin, 1977; Ruppel and Martin, unpublished), and amounts of BV accumulated by a single lesion are sufficient to limit fungal expansion (Martin, 1977). Thus, the rapidity and quantity of phytoalexin accumulation may contribute to a complex of responses that together determine the degree of

resistance exhibited by the host plant. Without exception, the diverse derived *Beta vulgaris* genotypes and phenotypes I tested produced the flavonoid phytoalexin BV in response to Cercospora leaf spot disease. Although this is not proof of BV's involvement in disease resistance, it certainly is a requirement if the compound is to be invoked as a resistance factor. Richardson (1981) found betavulgarin was synthesized by several *Beta* species after induction by a spore suspension of the non-pathogenic (to *Beta*) fungus *Helminthosporium carbonum;* however, BV was not detected in a few plants of *B. vulgaris* ssp. *maritima* ("from French seed," Richardson, 1981). Similarly, leaves of *Beta trigyna* from a botanic garden in London did not produce BV under *H. carbonum* elicitation, whereas *B. trigyna* plants grown from seed of two Russian populations accumulated BV. It is possible that the short elicitation time (48 hr) or comparatively insensitive techniques used (diffusion of compounds into spore suspension droplets on leaves, followed by organic extraction and thin-layer chromatography) might account for the lack of detectable phytoalexin production, or it simply could be that *H. carbonum* is not as effective as *C. beticola* in eliciting BV production. It is also possible that the populations in question do not have the ability to produce BV, or that they produce an alternative phytoalexin. At any rate, Richardson's negative finding suggests it will be worthwhile to continue to explore the ability of *Beta* species to produce B and BV when infected by *C. beticola.*

Harlan (1976) noted that when a defensive strategy against a disease-inducing fungus involves the production of fungistatic or fungitoxic compounds, there is a metabolic cost to the host. Thus, continuous selection for production of the secondary compounds is required. Smith and Gaskill (1970) showed that the genetic basis of resistance of sugarbeet to *C. beticola* is complex, involving a minimum of 4 or 5 gene pairs. The biosynthetic sequence leading to flavonoids also is complex, requiring the activation of several major biochemical pathways and the induction of appropriate enzymes. These observations suggest that there must have been considerable selection pressure to maintain this biochemical pathway in sugarbeet. In *Beta vulgaris (sensu lato),* a long coevolutionary relationship with *C. beticola* appears to have resulted in a dynamic balance between host and pathogen, and may be an underlying cause for the maintenance of flavonoid phytoalexin synthesis.

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