REGULAR ARTICLE

Control of spot blotch in barley plants with fungicide and Bauhinia variegata Linn. leaf extract

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Abstract

In barley plants (BRS-195), the disease detected as spot blotch is caused by Bipolaris sorokiniana, and is the most deleterious disease for the producers and the beer industry. For fungicides mediated control of this disease can cause risks to environment and human health. To eliminate these drawbacks, one of the methods considered is the use natural products. The purpose of the present study was to investigate the use of extract of leaves from Bauhinia variegata Link. and Opera® fungicide (active ingredient: pyraclostrobin) for controlling Bipolaris sorokiniana in barley plants. In Brazil there are two species of Bauhinia are seen, namely Bauhinia forficata and B. variegata. Extracts from B. variegata didn’t show fungitoxic action but B. forficata the action exist. The barley plant showed protection of 92 to 100% in local and systemic action. The chemical TLC assays showed the presence of phenols (rutin, coumaric acid, kaempferol) that can be related to a signal for activation of the defense responses against pathogen or mechanism of salicylic acid. Treatment with fungicide Opera® gave another mechanism and have only 60% of protection.

Key words: Barley, Bipolaris sorokiniana, Bauhinia variegata, Opera®

Introduction

In Brazil Barley has been used by beer makers for malting and for food purpose. During the barley development, several fungal diseases have been detected. Spot blotch is caused by Bipolaris sorokiniana (asexual fungi), and is the most serious deleterious diseases for the producers which affect the ears, darkening the grains and impairing the quality of malt and beer. The disease causes considerable losses in yield that can reach up to 30% of production. The infection depends on climatic conditions and ranges from 10 to 100% infection by the fungus. Another problem is the spots enlargement as the leaf grows and spread along the entire leaf blade and can be produce brown lesions. The producers have 100% losses because infected plant cannot produce normal heads (Teng, 1987; Agrios, 1988; Minella, 2001; Castro and Bach, 2004).

To prevent these losses, Opera® fungicide (active ingredient: pyraclostrobin) has been used to control disease but have a higher cost, risk of environmental contamination and intoxication during application for human. For eliminating these threats, alternative control measures can be adopted. The alternative control can be of induction of resistance in plants or, preventing or restricting the development and multiplication of the pathogen (Kuc, 1987). Inducers or elicitors of resistance have already been evaluated in the control of several diseases of plants with natural products (Guzzo et al., 1993; Benhamou, 1996; Gatz, 1997; Bach et al., 2003; Castro and Bach, 2004).

In Brazil, there are two species of Bauhinia one as ornamental plant, cultivated for afforestation and the other for manufacturing of wood wool board from Rio de Janeiro to Rio Grande do Sul. The two species can be confused because the flowers are similar but leaves and action in human health are different. Bauhinia forficata Link leaves extract was used with water as medicine for antidiabetic action in human (Arigony, 2005). B. variegata leaves extract showed the laxative action and used
against diarrhea (Asima and Satyesh, 1992). In morphology B. forficata has thorns, white flowers and the leaves present two lobes divided and, in adults the leaves can reach with 7 to 12cm in length. B. variegata has no thorns, flowers can be white or pink and, leaves have also two lobes but reach a smaller size (Miyake et al., 1986; Fortunato, 1986). The chemical substances are also different as evidenced by Engel et al. (2008) where B. variegata, didn’t show chemical marker as kaempferitrin but was present in B. forficata.

The aim of the present study was to investigate the effect of leaf extract of B. variegata and Opera® fungicide against B. sorokiniana infection in barley plants (cultivar Embrapa 195) under greenhouse conditions. For explaining the possible elicitor action, biochemical analyses were conducted in treated, untreated or infected leaves.

Materials and Methods
Suspension of pathogen
The pathogen used was B. sorokiniana obtained from infected barley leaves (Fundação Guarapuava-Agraria, Paraná) and kept in potato dextrose agar (PDA) medium on plates. After 10 days, conidia were removed by brushing the surface of the agar and material was suspended in 10 ml of sterile water followed by filtration through gauze. Concentration was adjusted to 10^5 conidia/mL and added Tween 20 (poly-oxyethylene sorbitan monolaurate, Sigma Chemical Co.) to a final concentration of 0.05%.

Biological control
Extracts from Bauhinia leaves were prepared with 1g of leaves in 5mL of water, filtered in whatman paper nº1. For biological control, one milliliter of each extract, in three dilutions, were incorporated in 5mL of culture medium PDA (Potato-dextrose-agar) submitted before to autoclave and after transferred in a slide of microscope and inoculated conidia of fungi (B. sorokiniana) and maintained in petri plates with humid and temperature of 27ºC. After 5 days, the area (cm²) occupied by the fungus was measured, and conidia were removed using 4mL of sterile distilled water and counted in a haemocytometer. Three replicates were made for each treatment.

Extracts from B. variegata (elicitor)
Leaves from B. variegata (pink flowers) were collected in Ibiuna, Sao Paulo and transported to the UNINOVE laboratory in a cooler. For extraction, 50g of leaves were ground in a blender with 250mL of distilled water. The homogenate was incubated in a refrigerator for one hour prior to being filtered through gauze and a 0.45µm Millipore filter. The filtered solution was then stored at -4°C until biochemical analysis and treatments. The concentrations of proteins (Lowry et al., 1951) and phenols (Swain and Hillis, 1959) were quantified as part of the biochemical analysis.

Preparation of barley plants and induced local protection
Barley plants (Embrapa BRS195 – from Foundation Agraria, state of Paraná), were grown from ten seeds in clay pots (15cm diameter) filled with a fertilized soil (red soil with NPK 10:10:10 with micronutrients) and maintained in a greenhouse under 12h photoperiod (approximately 190IE/m²/s) for around 3 weeks, until they were in stage 5 (tillering) (Large, 1954).

Groups of 10 plants were used in each treatment. Each treatment was replicated three times and data were submitted to variance analysis. Plants were arranged in a complete randomized block design and the combination of challenger and protector in each treatment. Around 10mL of the conidia suspension, or extract of elicitor or water were used in each treatment. Treatments were: (a) healthy (plants sprayed with water); (b) Inducer: plants sprayed with elicitor extract in two dilutions (0.535mg of proteins and 0.267mg of proteins); (c) pathogen inoculated (plants pulverized with conidia suspension of pathogen); (d) Inducer-pathogen and in two dilutions: inducer-treated and after 24 h inoculated with conidial suspension; e) ditto the group d, however, after 48 hours; f) ditto the group d, however, after 72 hours; g) plants pulverized with Opera® fungicide (pulverized with 2mL of a 200 fold dilution of product in water) and afterwards inoculated with a conidial suspension. Plants from groups d, e, f were initially pulverized with the elicitor, and after 24, 48 and 72 hours, at room temperature and 12-hour photoperiod (fluorescent light 7.35 W m⁻²), the leaves were inoculated with conidia suspensions, by pulverization. During the first 24 hours after the inoculation of the pathogen, all plants were kept in a humid chamber (80% relative humidity), at room temperature and in the dark. After that, plants were transferred to the greenhouse and kept at room temperature and 12 hours of light per day (Bach et al., 2003).

Preparation of barley plants and systemic resistance
In another group of plants the systemic protection assay was performed using the same cultivar and the elicitor extract in the dilution that have a positive reaction in local protection. Three leaves were market and the first leaf were the
oldest, the second leaf intermediary and the thirty a new leaf. The second leaves of barley plants were coated with the elicitor extract (Syst L2T) and the first and thirty were coated with water. After 48 hours, all the leaves were submitted to total pulverization with suspension of the conidia. Healthy groups were pulverized using water. Protection level was evaluated 7 days after the inoculation of the pathogen, based on the number of infected leaves in ten plants (Bach et al., 2003). First, second and thirty leaves was removed, separated and submitted to extraction.

**Extraction of barley leaves**

Barley leaves from all experiments were performed in duplicate and analyzed by the Student’s test. One gram of the leaves were ground in presence of 1mL of cold phosphate buffer (pH=7, 0.05 mol/L). After one hour incubation at 4°C, each extract was filtered through gauze and then quantified proteins and phenols. The amount of protein in sample was estimated using Lowry method (Lowry et al., 1951), based in a standard curve of standard protein solution as BSA (mg bovine serum albumin/mL- Sigma) and absorbance were determinate using a spectrophotometer Fenton with software. For phenols the method used was based in Swain and Hill (1959) with standard curve prepared with chlorogenic acid (mg/mL) (Sigma).

From plants healthy, BAU control, infected, treated with Opera®, BAU 48h and BAU 72h, one gram of leaf material was ground in methanol and 10µL of each samples was subjected to thin layer chromatography (Merck) and separated with organic phase from Butanol-acetic acid-water (BAW 4:1:5). Spots were visualized with UV light and ferric chloride (1% in alcohol). Areas from bands were measure in program CP Atlas. Standard samples used for DC-Meth was kaempferitrin (RF=0.36) and for BAW was rutin (RF=0.48), kaempferol (RF=0.94) and o-coumaric acid (RF=0.68).

**Results and Discussion**

**Morpho-anatomical diagnosis**

The preliminary investigation was morpho-anatomical diagnosis from leaves and flowers present in two species from the genus *Bauhinia*, that have peculiar bilobate leaves, which render the common name pata-de-vaca (cow’s hoff) and were used as medicinal plants. *B. variegata* presented leaves whose two lobes are sharply rounded apex, which differs from the apex acute *B. forficata*. Flowers from *B. forficata* were white and in *B. variegata* can be white and pink (Miyake et al., 1986; Fortunato, 1986; Shah et al., 2010).

**Biological control**

The quantification of concentration of proteins and phenols was: for *B. variegata* was present 5.35mg of proteins and 0.28mg of phenols while for *B. forficata* was 1.25mg of proteins and 3.8mg of phenols. Extract from *B. forficata* was preliminary concentrated in dialysis membrane (MM3000) against carbowax (PEG-poliglicol MM6000), maintained in refrigerator by 10 hours. The proteins were obtained eight times more concentrated and then diluted with water at same concentrations from *B. variegata* and used for test involved biological control in four concentrations.

The results in biological control above the *B. sorokiniana* showed that aqueous extract of *B. forficata* inhibited the fungal development and conidial production that can be considered as fungitoxic action and the extract of *B. variegata* showed no difference in the development and production of conidia when compared to the control slide because of lack of biological control above the fungi (Table 1).

Accordingly with Georgopoulos (1984) when worked with adaptation of fungi to fungitoxic compounds that can be concluded that some fungitoxic chemicals often fail to protect crops because the target fungi develop resistance. So, in this work we used *B. variegata* (pink flower) because the extract didn’t presented fungitoxic action against fungi.
Table 1. Development and production of conidia from *B. sorokiniana* submitted to different concentrations of proteins from aqueous extracts from *B. variegata* and *B. forficata*.

| Extracts  | dilution of extract (concentration of proteins mg) | Number of conidia x 10^4* | Total area (cm)* |
|-----------|---------------------------------------------------|---------------------------|-----------------|
| *B. variegata* | 2.00 | 2.0±0.05 | 2.0±0.02 |
| *B. forficata* | 2.00 | 0.1±0.004 | 0.6±0.03 |
| *B. variegata* | 1.07 | 2.0±0.03 | 2.0±0.02 |
| *B. forficata* | 1.07 | 0.1±0.003 | 0.8±0.02 |
| *B. variegata* | 0.50 | 1.9±0.03 | 2.0±0.03 |
| *B. forficata* | 0.50 | 0.2±0.002 | 1.0±0.04 |
| *B. variegata* | 0.26 | 1.9±0.004 | 2.0±0.02 |
| *B. forficata* | 0.26 | 0.3±0.003 | 1.2±0.03 |
| Control (*B. sorokiniana*) | X | 2.0±0.08 | 2.0±0.03 |

* Media of three repetitions with±SD. Same letters in columns was not different statistically when compared with control. Different letters in columns were different statistically compared with control (student T test).

**Induced of local resistance**

The percentage of local protection was evaluated at two concentrations of the inducer (aqueous extract from *B. variegata*) containing 0.535mg and 0.267mg of protein. At dilution with 0.535mg proteins the protection ranged from 92 to 100% while with 0.267mg of proteins the protection ranged from 78 to 100%.

The results were compared with those of the treatment with the fungicide with the level was 60% (Table 2). It is interesting to note that plants treated with inducers and challenge presented more protection than a plant with fungicide. In biochemical analyses, barley plants treated with elicitor at all intervals of time and plants treated with fungicide, showed higher amounts of protein, lower concentration of phenols when compared with infected plants. For example, in period of 72h inducer-challenge, the protection and concentration of protein was higher but quantity of phenols was decreased when compared with untreated plants. For example, in period of 72h inducer-challenge, the protection and concentration of protein was higher but quantity of phenols was decreased when compared with untreated plants. Infected plants presented more concentration of phenols and decreased protein when compared with treated plants.

**Induced of systemic resistance**

Systemic protection against *B. sorokiniana* was also obtained when aqueous extract from *B. variegata* (with 0.535mg of proteins) was applied 48h before challenge inoculation (Table 2). The higher protection occurred with upward effect but a minor action also occurred in lower leaf. In 72h between inducer-challenge, in the third leaf (young leaf) presented 90%, the leaf 2 that was treated presented 99% and the leaf 1 (oldest leaf) presented 100% of protection. The same effect occurred in other periods from 24h and 48h. This demonstrates that even if the inducer is applied at the bottom of the plant, its effect will move to the upper leaves. This effect was also observed by Bach et al. (2003), Castro and Bach (2004) in work with other elicitors.

In systemic treatments, the leaf that received inducer (L2T) presented higher concentration of protein when compared with leaf 1 and 3. For induced protection, the leaf 2 presented higher protection as the leaf 3 showing action upward. Other direction downward also presented protection but that was decreased when compared with the leaf 1 (Table 2, 3). Presence of higher concentration of proteins was associated with the action of induced resistance because the elicitors are responsible for triggering a signal in the host when occur the attack of a pathogen. These results were also in par with other authors (Hwang and Kim, 1990; Du and Wang, 1992; Anuratha et al., 1996; Benhamou, 1996; Manandhar et al., 1999; Kuc, 2001; Kombrink and Schmelzer, 2001) (Table 2, 3).
Table 2. Number of leaves with spot blight caused by *B. sorokiniana* and percentage of protection in barley leaves (Embrapa 195), using aqueous extract of *B. variegata* (pink flower) as inducer.

| Treatments   | mg proteins | number of total leaves | number of infected leaves | % protection |
|--------------|-------------|------------------------|----------------------------|--------------|
| Healthy      |             | 20 X                   | X                          |              |
| Infectada    |             | 20 19                  | 0 a*                       |              |
| Opera®       |             | 20 8                   | 60±1 h**                   |              |
| BAU C        | 0.535       | 81 0                   | 100 b,b*                   |              |
| BAU 72h      | 0.535       | 81 3.2                 | 96±0.5 c, f                |              |
| BAU 48h      | 0.535       | 81 7.2                 | 92±1 d, g                  |              |
| BAU 24h      | 0.267       | 20 0                   | 100 b,b                    |              |
| BAU 72h      | 0.267       | 20 3.4                 | 83±0.8 c,h                 |              |
| BAU 48h      | 0.267       | 20 4.5                 | 78±0.5 e,i                 |              |
| Syst BAU 72h |             | 20 2                   | 90±1c**                    |              |
| Syst BAU 48h |             | 20 0.2                 | 99±0.4 b                   |              |
| Syst BAU 24h |             | 20 0                   | 100 a                      |              |

Groups: Healthy (plants sprayed only with water); Infectada (plants sprayed only with conidial); Opera® (0.1mL from product diluted in 20mL of water and used in pulverization only 2 mL/plant); BAU C (plants sprayed only with extracts of *B. variegata* in two dilutions); BAU 72h (plants sprayed with extract (two dilutions) of the inductor and 72h after inoculated with pathogen); BAU 48h (ditto with the previous range of 48 hours); BAU 24h (ditto with the previous range of 24h); Syst BAU 72h L1(old leaf) : coated the first leaf with 2 mL of water; L2T: coated the second leaf with 2 mL of the extract of the inductor; L3(new leaf): coated the third leaf with 2 mL of water. After 24, 48 and 72 were inoculated the total plant with conidia of *B. sorokiniana*. *media of percentage of protection of total of 10 plants per treatment from three experiments ±SD. Mean values with different first letter, statistically significantly different from the infected plants (P<0.05); and when the second letter was the same have not difference statistically between treatments but when these letters was different statistically was number different between treatments (P<0.05), according to the students t-test and Origin (Anova). **Media of percentage of systemic protection of total of 10 plants + SD and compared with infected plants and with extract of Bauhinia. Value was different statistically from all plants submitted to systemic protection.

Figure 1. Rf (mobility) and band area (mm²) from extract of Bauhinia variegata (pink and white flower) and *B. forficata*. Samples with 10 μL. Reagent: dichloromethane-methanol (7:3) for Kaempferitrina (Kaempf Rf=0.36). Reagent BAW (4:1:5 organic phase), standard Rutin (Rf=0.48), Kaempferol (Kaemp Rf=0.94) and coumaric acid (coumacid Rf=0.68). Spots revealed with UV and ferric chlorite 1% and area measure program CP-Atlas.
Table 3. Concentration of protein (mg SAB), and phenols (mg chlorogenic acid), present in leaf extracts of barley plants (cultivar Embrapa 195) after treatment with aqueous extract of B. variegata, Opera®, water against B. sorokiniana.

| Treatments | Conc. proteins in extract | Proteins (mg BSA/mL)* | Phenols (mg clorog ac/mL)* |
|------------|---------------------------|----------------------|---------------------------|
| Healthy    | 0.487±0.04 b              | 0.10±0.02b           |                           |
| Infected   | 0.120±0.01 a              | 0.86±0.20a           |                           |
| Opera®     | 0.320±0.04b               | 0.13±0.01 b          |                           |
| BAU C      | 0.535                     | 0.468±0.06b          | 0.03±0.005c               |
| BAU 72h    | 0.535                     | 0.575±0.04b          | 0.03±0.005c               |
| BAU 48h    | 0.535                     | 0.498±0.04b          | 0.05±0.005c               |
| BAU 24h    | 0.535                     | 0.471±0.03b          | 0.05±0.004c               |
| BAU C      | 0.267                     | 0.445±0.05b          | 0.03±0.002c               |
| BAU 72h    | 0.267                     | 0.420±0.04b          | 0.03±0.002                |
| BAU 48h    | 0.267                     | 0.431±0.04b          | 0.06±0.003c               |
| BAU 24h    | 0.267                     | 0.417±0.05b          | 0.08±0.002                |
| Syst BAU 72h |                      |                     |                           |
| L1         | 0.480 b                   | 0.05 c               |
| L2T        | 0.520 b                   | 0.03 c               |
| L3         | 0.500 b                   | 0.08 c               |
| Syst BAU 48h |                      |                     |                           |
| L1         | 0.385 b                   | 0.06 c               |
| L2T        | 0.450 b                   | 0.06 c               |
| L3         | 0.480 b                   | 0.09 c               |
| Syst BAU 24h |                      |                     |                           |
| L1         | 0.350 b                   | 0.08 c               |
| L2T        | 0.410 b                   | 0.09 c               |
| L3         | 0.430 b                   | 0.10 b               |

Groups: Healthy (plants sprayed with water); Infected (plants sprayed only with conidial); Opera® (0.1mL from product diluted in 20mL of water and used in pulverization only 2 mL/plant); BAU C (plants sprayed only with elicitor in two dilutions); BAU 72h (plants sprayed with elicitor 72h (with 2 dilutions) and after inoculated pathogen); BAU 48h (ditto with the previous range of 48 hours), BAU 24 (ditto with the previous range of 24); SYST: L1: Strokes the first sheet (F1) with 2 mL of water L2T: Strokes the second sheet (F2) with 2 mL elicitor; L3: Strokes thirty leaf (F3) with 2 mL of water. After 24, 48 and 72 were inoculated with conidia of Bipolaris sorokiniana in all leaves. * Averages involving three replicates of each test + SD. Means with different letters in columns differ significantly at 0.05% (Student t-test `s) when compared with infected plants. In Syst treatments don’t have SD because the numbers are very little about 0.0001 or 0.0002.

Chromatography from extracts of Bauhinia

To prove that in work was used B. variegata, were used a test thin layer chromatography with two reagents. Engel (2008) observed that B. forficata in reagent DC-Meth showed a band of Rf=0.36 and that is a marker for presence of kaempferitrina. In the results from present work was observed that B. variegata both with pink or white flowers did have not that band.

With reagent BAW (organic phase) B. variegata pink flower showed three bands with Rf 0.15; 0.48 and 0.68 but the white flower had four bands with Rf 0.15, 0.48; 0.68; 0.94. The band with Rf=0.15 is a marker for all varieties from Bauhinia. Extract from B. variegata pink and white flower present the same band with Rf=0.48 that coincide with rutin standard (Figure 1).

Ferrer et al. (2008) and Victório et al. (2009) described flavonoids are metabolites produced as part of plant defense, especially against the effects of ultraviolet radiation and their contents are in greater concentration in the leaves that is the main part of plants exposed to solar incidence. The rutin and Kaempferol in cassava increased with defense responses to diseases (Tanaka et al., 1983; Buschmann et al., 2000). The coumaric acid is related to resistance for produce salicylic acid and that can be related to inducer of resistance or mechanism of resistance.

Chromatography from extracts of Barley plants

TLC analysis of a healthy barley plant extract included three bands and infected plant with B. sorokiniana presented only one band. The other two bands from infected leaves perhaps were used or degraded by fungus.

With barley plant (treated only the elicitor BAU) present five bands but three were equal in area and Rf to bands obtained from healthy plants. The other two bands correlated with p-coumaric acid and rutin (Table 4). It is interestingly that p-coumaric acid and rutin is likely come from elicitor extract.

Barley plants treated with Bauhinia extract and sprayed with conidia suspension, presented three bands correlated to standards o-coumaric acid, p-coumaric acid and benzoic acid. The band of p-coumaric acid in treated plants presented an area
equal to 400mm² and in control plants, an area equal to 590mm² indicating that only some of the p-coumaric acid penetrated into the plant in 48 and 72h (Table 4). Area of o-coumaric acid increased over time and correlated to increases protection.

Table 6. Bands observed in TLC following barley treatments.

| Treatments | Rf    | Area * | Standard** |
|------------|-------|--------|------------|
| Healthy    | 0.428 | 82     |            |
|            | 0.560 | 4069   |            |
|            | 0.710 | 2691   |            |
| Infected   | 0.440 | 8685   |            |
| Opera (48h)| 0.539 | 2793   |            |
|            | 0.586 | 1163   |            |
|            | 0.763 | 1186   |            |
|            | 0.839 | 1318   |            |
|            | 0.915 | 163    |            |
|            | 0.991 | 661    |            |
| BAU C      | 0.428 | 82     |            |
|            | 0.480 | 420    | Rutin      |
|            | 0.560 | 4069   |            |
|            | 0.680 | 590    | p-coum acid|
|            | 0.710 | 2691   |            |
| BAU 48h    | 0.420 | 58     |            |
|            | 0.560 | 1852   |            |
|            | 0.620 | 1580   | o-coum acid|
|            | 0.680 | 400    | p-coum acid|
|            | 0.740 | 2060   | benz acid  |
| BAU 72h    | 0.420 | 38     |            |
|            | 0.560 | 1022   |            |
|            | 0.620 | 2950   | o-coum acid|
|            | 0.680 | 400    | p-coum acid|
|            | 0.740 | 4050   | benz acid  |

*Area (mm²)
**Bands correlated to standard

Raskin (1992) and Ribnicky et al. (1998) explained that salicylic acid (SA) is a signal in systemic acquired resistance and as an inducer of the alternative protein in tobacco cell suspensions. The occurrence of SAR (systemic acquired resistance) in response to a pathogen requires a long-distance transport of a factor originating in the tissue expressing the hypersensitive response that moves systemically to other parts of the plant (Chong et al., 2001; Dmitriev, 2003; Sequeira, 1979, 1983). It was suggested that SA is responsible for SAR plants. In this work the results demonstrated that in B. variegata (pink flower) have cumaric acid, rutin and kaempferol that’s related to a signal for the activation the defense responses against pathogen or mechanism of salicylic acid.

In barley plants treated with Opera®, six bands were identified but none correlated to the standards used in work and possible the mechanism was different.

**Conclusion**

In conclusion, extract of B. variegata acted as elicitor of local and systemic resistance (ascending higher than in the downswing) in barley plants against Bipolaris sorokiniana upon 90% of protection. Presence of inducer was correlated with increased protein, decreased phenols and presence of o-coumaric acid. This suggests that the mechanism of protection involves SA biosynthesis. With barley plants treated with fungicide Opera® and later challenged with conidia from same fungi, demonstrated 60% of protection and mechanism is probably different because don’t have o-coumaric acid.

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