Table 15 : Level of phenolics in healthy and B. carbonum infected tea leaves of resistant and susceptible varieties.

Variety	Phenols (mg/g fresh wt. leaf tissue)			
	Total phenol		Orthodihydroxy phenol	
	H	I	H	I
Susceptible				
TV-18	4.4	4.2 (-4.5)	0.24	0.20 (-16.7)
TV-9	4.2	4.15(-1.2)	0.23	0.19 (-17.4)
TV-17	4.7	4.3 (-8.5)	0.20	0.18 (-10.0)
Resistant				
TV-26	4.8	5.4 (+11.1)	0.33	0.42 (+27.3)
TV-25	5.2	5.5 (+5.7)	0.28	0.39 (+39.3)
TV-16	4.9	5.5 (+12.2)	0.31	0.42 (+35.5)

H = Healthy ; I = Inoculated with B. carbonum

** = After 48 hour of incubation.

* = Value given in parentheses are percent decrease (-) or percent increase (+) in relation to healthy.

Part V : Detection of antifungal compounds in tea leaves after challenge with B. carbonum

Phytoalexin production is known to be one of the conferral mechanisms of disease resistance in several plants (Cruickshank, 1963 ; Ingham, 1972 ; Keen, 1981 ; Purkayastha,1985;

Ebel and Grisebach, 1988 ; Madamanchi and Kuc, 1991). The differential resistance of tea varieties in response to infection with B. carbonum may be attributed to differences in their abilities of phytoalexin production. Since no work on tea phytoalexin has been reported so far, it was considered worthwhile to investigate whether any antifungal substance (phytoalexin) was involved in the differential disease reaction in the tea varieties against B. carbonum.

At the onset, biological activities of exudates and diffusates of tea leaves were tested. Drop diffusate method was followed to collect leaf exudate and diffusates from six tea varieties three each from resistant (TV-26, TV-25, TV-16) and susceptible (TV-18, TV-9, TV-17) plants after 48 h of incubation as described in Materials and methods. Their biological activities were evaluated on spore germination and germtube growth of B. carbonum. The results in Table-16 indicate that the diffusates collected from the resistant tea varieties (TV-26, TV-25 and TV-16) were more fungitoxic than those from the susceptible varieties (TV-18, TV- 9 and TV-17). An interesting observation made in this experiment is that tea leaf exudates also contained some fungitoxic substances.

The results from the drop diffusion technique (Table-16) strongly suggest that diffusates obtained from all the six varieties of tea leaves contained some antigungal compounds.

Table 16 : Effect of leaf exudate and diffusate of resistant and susceptible tea varieties on germination and germ tube growth of *B. carbonum*.

Varieties	Treatment[a]	Germination[b] percentage	Mean germ[c] tube length (µm)
Resistant			
TV-26	Exudate	57.0	108.7
	Diffusate	24.0 (-57.90)	56.9 (-47.6)
TV-25	Exudate	66.0	130.0
	Diffusate	29.0 (-56.06)	71.0 (-45.2)
TV-16	Exudate	76.0	122.0
	Diffusate	35.5 (-53.90)	79.0 (-35.2)
Susceptible			
TV-18	Exudate	97.6	266.5
	Diffusate	75.5 (-22.6)	138.1(-48.2)
TV-9	Exudate	96.0	249.0
	Diffusate	76.3 (-20.5)	176.0(-29.31)
TV-17	Exudate	97.0	263.0
	Diffusate	79.0 (-18.5)	177.0(-32.69)
Distilled water (control)		98.4	316.5

a Exudate and diffusate collected after 48 h.

b Average of 500 spores

c Average of 50 germ tubes

Note- Values in parentheses indicate percentage reduction (-) in relation to Exudate

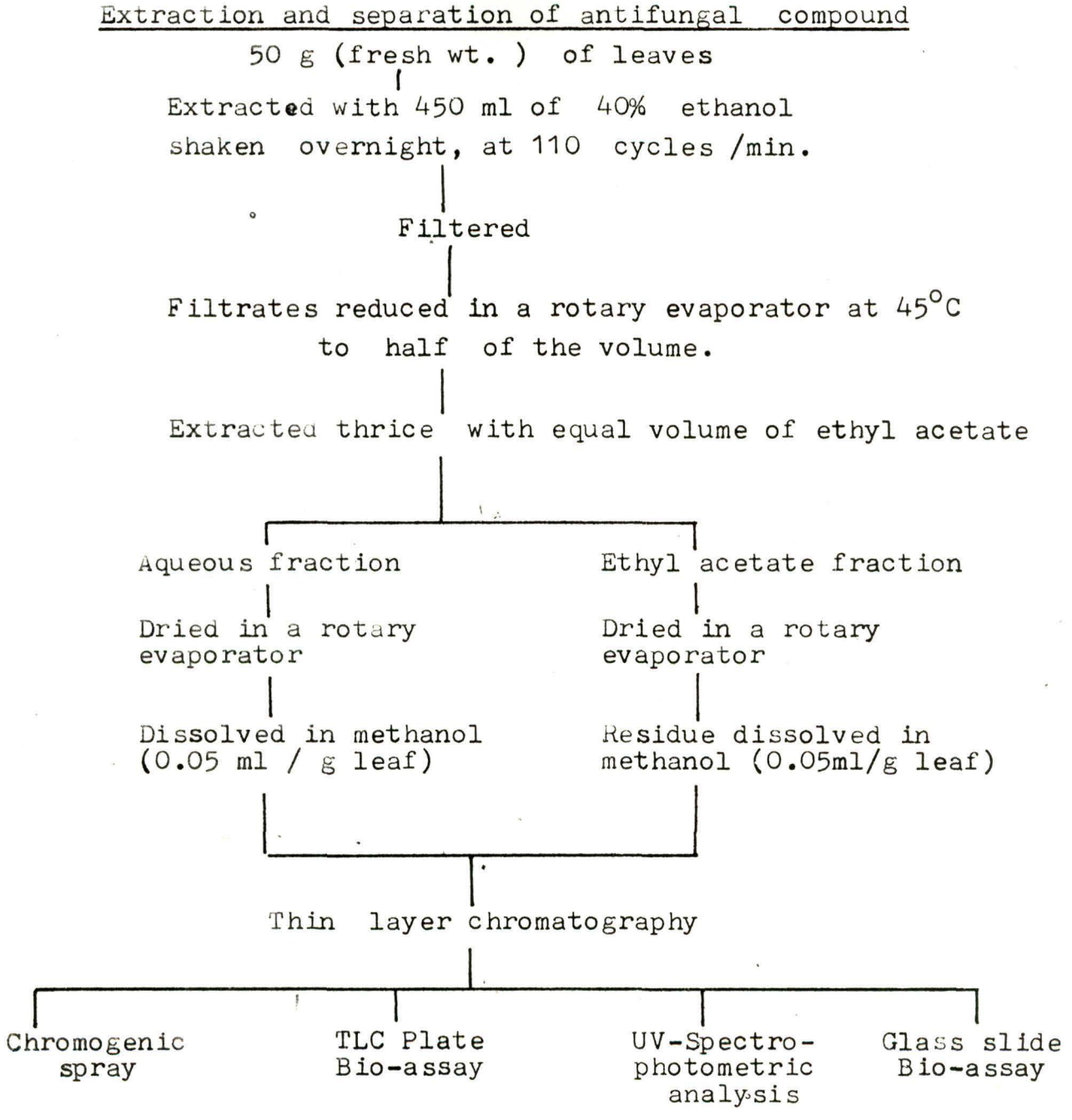
However, this method is not easily applied to large amount of tissues , and the utility of this technique is diminished further

by the relatively low solubility of many phytoalexins in pure water. Hence, further experiments were carried out following facilitated diffusion technique as suggested by Keen (1978), for the detection of antifungal substance (phytoalexin) from relatively large samples of freshly harvested tea leaves inoculated with B. carbonum.

Antifungal compounds were extracted separately from the healthy and inoculated (with B. carbonum) leaves of resistant (TV-26 and TV-25) and susceptible (TV-18 and TV-9) varieties of tea after 48 h of inoculation following the scheme given below.

Aliquots of water fraction and ethylacetate fraction of healthy and inoculated tea leaf extracts were developed initially in two different solvent systems, viz. Chloroform : Methanol (9:1 v/v) and Hexane : Ethyl acetate : Methanol (60:40;1 v/v). Finally chloroform-Methanol (9:1) was chosen as the best solvent on the basis of their better separation.

TLC plates after spraying separately with Folin Ciocalteau's reagent, $FeCl_3$ - K_3FeCN_6, diazotized p-nitroaniline and Vanillin - H_2SO_4 gave positive colour reaction on the silica gel layer indicating the presence of phenolic compounds in ethylacetate fractions of both healthy and infected leaves of the said four tea varieties.


Extraction and separation of antifungal compound
50 g (fresh wt.) of leaves
Extracted with 450 ml of 40% ethanol
shaken overnight, at 110 cycles /min.
Filtered
Filtrates reduced in a rotary evaporator at 45°C
to half of the volume.
Extracted thrice with equal volume of ethyl acetate
Aqueous fraction
Ethyl acetate fraction
Dried in a rotary evaporator
Dried in a rotary evaporator
Dissolved in methanol (0.05 ml / g leaf)
Residue dissolved in methanol (0.05ml/g leaf)
Thin layer chromatography
Chromogenic spray
TLC Plate Bio-assay
UV-Spectro-photometric analysis
Glass slide Bio-assay

These crude extracts (water fraction and ethyl acetate fractions) were bioassayed for antifungal compounds using B. carbonum as the test organism following TLC plate bioassay technique as described in detail under materials and methods. Two compounds (A and B) were fungitoxic from ethyl acetate fraction as determined by the "On-the-Chromatogram-inhibition-Assay" (Plate-IX, figs. 1 & 2). The inhibitory compounds were present not only in leaf extracts of inoculated resistant (TV-25 and 26) and susceptible (TV-9 and 18) varieties but also in extracts of noninoculated healthy leaves. Water fraction from these varieties did not exhibit any antifungal compound on the chromatogram inhibition assay.

Healthy extracts of all four varieties (TV-9 , 18, 25 and 26) showed a prominent inhibition zone at Rf 0.8. There was no evidence of the inhibition zone-A after 48 h of inoculation in TV-9 and 18, whereas traces of this inhibition zone was still evident in inoculated leaves of TV-25 and 26. In the extracts from inoculated leaves of both susceptible and resistant varieties inhibition zone-B appeared at Rf 0.65. Diameter of the inhibition zones exhibited on the chromatogram inhibition assay and its Rf values are presented in Table-17. Appearance of above two inhibitory compounds A and B was confirmed by three repeated experiments.

Silica gel corresponding to these inhibition zones

PLATE - IX (figs. 1-6) : TLC-plate bioassay of ethylacetate extracts of healthy and infected tea leaves, 48 h after inoculation with B. carbonum.

fig.1 - Healthy (Left) and inoculated (right) leaf extracts (TV-18)

fig.2 - Healthy (Left) and inoculated (right) leaf extract (TV-26)

fig.3 - Nickel chloride treated, uninoculated leaf extract (TV-18)

fig.4 - Untreated, inocula ed leaf extract(TV-18)

fig.5 - Nickel chloride treated, inoculated leaf extract (TV-18)

fig.6 - Untreated, inoculated leaf extract (TV-26)

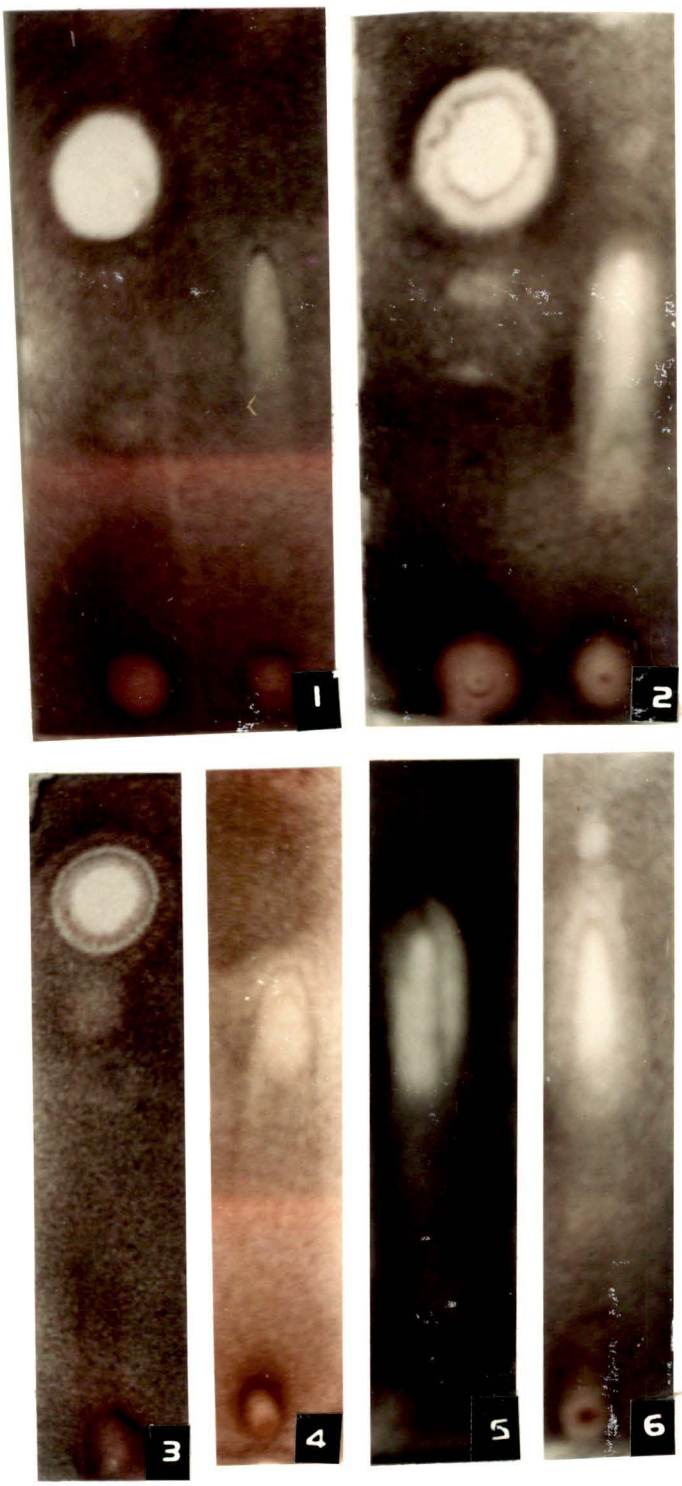


PLATE - IX

Table 17 : TLC - plate bioassay of antifungal compounds

Varieties	Treatment	Diameter of the inhibition zone (mm)	
		Compound-A (Rf= 0.80)	Compound-B (Rf= 0.65)
Resistant			
TV - 26	Healthy	22.5	4.2
	Infected	5.5	11.2
TV - 25	Healthy	22.0	4.2
	Infected	6.3	9.8
Susceptible			
TV - 18	Healthy	18.2	0
	Infected	0	4.0
TV - 9	Healthy	18.6	0
	Infected	0	5.1

Solvent system - Chloroform-Methanol (9:1,v/v)
Organismtested - Bipolaris carbonum
Incubation period - 72 h.

were removed from TLC plates and eluted by methanol. The purified eluants obtained after rechromatography were tested for antifungal activity by the spore germination assay as described in detail under materials and methods. In case of healthy extracts, compound - A showed the highest fungitoxic activity in the spore germination assay. This antifungal compound showed brown colour reaction when sprayed with vanillin-H_2SO_4 at the Rf 0.8 (Table-18) which corresponds with the Rf value and colour reaction authentic catechin

Table 18 : Colour reaction of the antifungal compounds in visible light after spraying with chromogenic reagents.

Chromogenic spray	Colour reaction of antifungal compounds	
	A (Rf = 0.8)	B (Rf = 0.65)
Vanillin-H_2SO_4	Brown	-
Folin Ciocalteu's reagent	-	Deep blue
$FeCl_3$-$K_3Fe(CN)_6$	-	Blue
Diazotized p-nitroaniline	-	Brick red

Solvent system - Chloroform-methanol (9:1, v/v)

Compound - B showed the highest fungitoxic activity in resistant varieties (TV-25 and 26) after 48 h of inoculation (Table 19) in comparison to the susceptible varieties (TV-9 and 18). Partially purified antifungal compound-B was assayed for toxicity against six additional fungal species (viz. Colletorichum camelliae, Pestalotiopsis theae, Helminthosporium oryzae, H. maydis and H. sativum). Results (Table 20) revealed that the compound B inhibited the germination of all the fungal species tested. Thus antifungal nature of the compound B was confirmed. The antifungal compound B showed positive colour reaction of phenolics with the chromogenic sprays as mentioned in Table-18.

Table 19 : ED_{50} value of the isolated antifungal compounds from different varieties of tea.

Varieties	Treatment	ED_{50} value [a]	
		Compound A (Rf= 0.80)	Compound B (Rf= 0.65)
TV -26	Healthy	4.2	15.2
	Infected	10.8	5.6
TV -25	Healthy	5.0	16.1
	Infected	11.6	6.3
TV -18	Healthy	7.2	34.6
	Infected	-- [b]	18.6
TV -9	Healthy	8.4	38.9
	Infected	-- [b]	16.8

[a] values are equivalent to mg fresh wt. of tea leaves

[b] not detected

Table 20 : Inhibitory effect of compound B on spore germination of different fungus.

Fungal species	Percent inhibition [a] Compound B (50 µg/ml)
Pestalotiopsis theae	92
Colletotrichum camelliae	89
Helminthosporium oryzae	94
H. maydis	80
H. sativum	73

[a] On the basis of 100 spores

Partially purified (by preparative TLC) compound B was examined in an UV- spectrophotometer (Model-Shimadzu -160) and absorption peaks at 214 nm and 276 nm were recorded. The compound B was identical to an authentic sample of catechol, as determined by thin layer chromatography and UV - spectrophotometry (Fig 9). Hence, quantification of the antifungal compound B was done from UV-spectrophotometric curve by considering molar extinction coefficient of authentic catechol 6,000 at 214 nm (Williams and Fleming, 1988).

$$\text{\# Molar extinction co-efficient } (E) = \frac{\text{O.D. of the tested solution}}{\text{Concentration}(x)^{*} \text{ of the tested solution (moles/litre)} \times \text{Path length of the cell (cm)}}$$

* moles/litre converted to g/litre by multiplying moles with molecular weight of catechol. Results have been expressed in µg/g fresh wt. of leaves.

\# Anonymous (1980)

Antifungal compound B accumulated in two resistant (Tv-25 and 26) and two susceptible (TV-9 and 18) varieties of tea after 48 h of inoculation was estimated and compared with their healthy controls. It appears from the result (Table 21) that in inoculated leaves greater amount (439 - 510 µg/g fresh wt.) of antifungal compound B (catechol) accumulated in the resistant varieties in comparison to that in the susceptible varieties (187-212 µg/g fresh wt.) concentration of this compound

UV-SPECTRA OF AUTHENTIC CATECHOL AND ANTIFUNGAL COMPOUND-B EXTRACTED FROM TEA LEAVES (Tv-26) INOCULATED WITH B. carbonum.

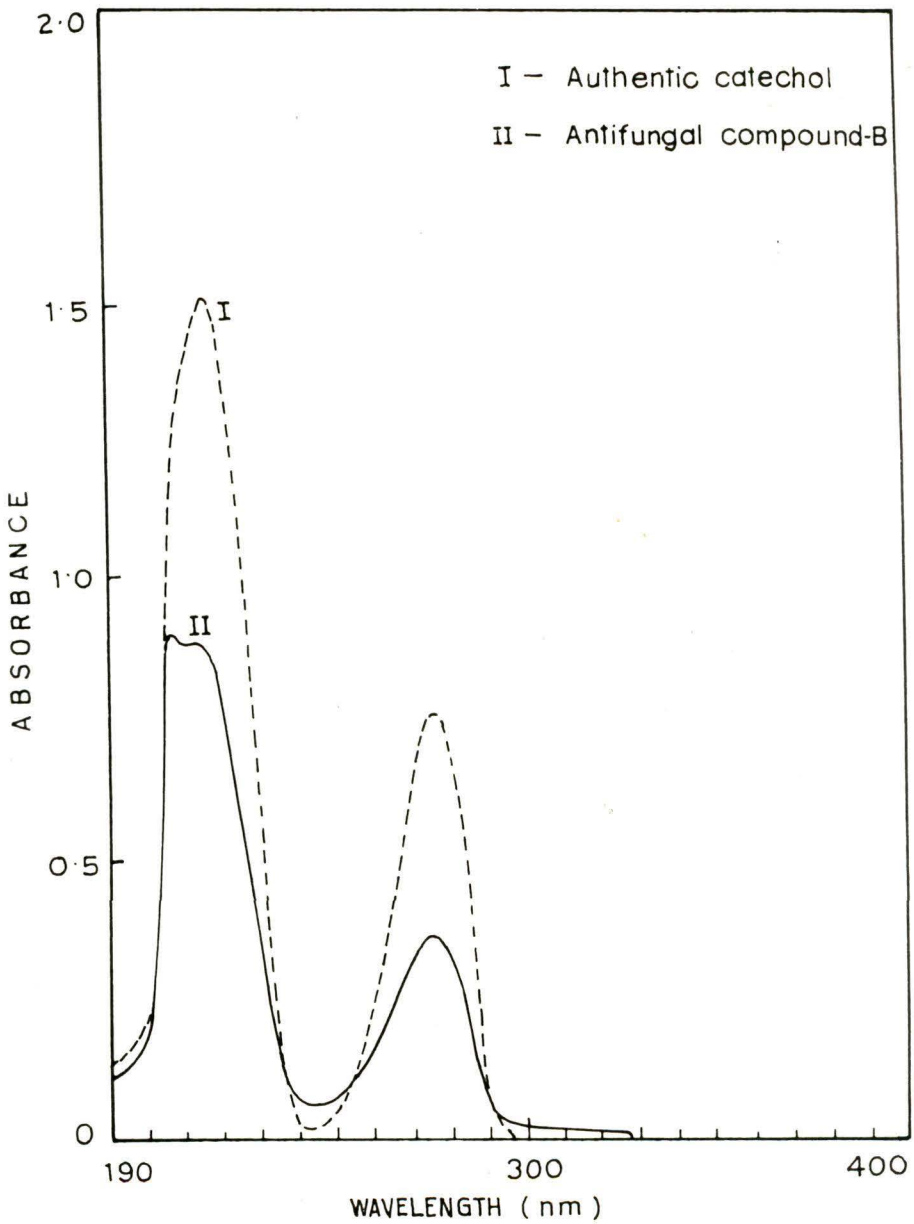


FIG. 9.

Table 21 : Quantitative estimation of antifungal compound B in resistant and susceptible tea varieties inoculated with B. carbonum.

Tea varieties	Compound - B[a] (µg/g fresh weight of leaves)	
	Healthy	Infected
Resistant varieties		
TV - 26	88	510
TV - 25	62	539
Susceptible varieties		
TV - 18	45	187
TV - 9	57	212

[a] Extraction of compound B was done after 48 h of inoculation.

in healthy leaf tissues were very low (45 - 88 µg/g gresh wt.)

Part VI : Experiments on the alteration of disease reaction by the application of various chemicals and their fungitoxic effects on B. carbonum. in vitro.

It is evident from the result presented in Part V that resistant varieties accumulated greater amount of the antifungal compound B than the susceptible varieties in response