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Study on the Relationship between Secondary Metabolites and Resistance to Anthracnose in *Stylosanthes*

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Abstract: *Stylosanthes* is an excellent tropical leguminous forage. Anthracnose caused by *Colletotrichum gloeosporioides* is a major limiting factor in *Stylosanthes* production, which can lead to reduced forage and seed production and decreased quality. How to quickly screen resistant parents and varieties of *Stylosanthes* that are resistant to anthracnose has become an urgent problem to be solved in production. In this study, the high-performance liquid chromatography detection technology was established for simultaneous analysis of the content of salicylic acid, triethyl orthobenzoate, indazole, and soyasapogenol B in *Stylosanthes* leaves. The technology was further applied in the analysis of the relationship between secondary metabolites and resistance to anthracnose in 105 accessions of *Stylosanthes*. The result shows that salicylic acid is the most important factor for resistance to anthracnose. By detecting the content of salicylic acid in *Stylosanthes* leaves, a rapid screening technology for anthracnose-resistant parents and varieties of *Stylosanthes* was established, and 5 accessions of *Stylosanthes* with high resistance to anthracnose were screened. This study is of great significance to the *Stylosanthes* germplasm preservation and identification and its breeding.

Keywords: *Stylosanthes spp*.; Secondary metabolites; Resistance to anthracnose; Germplasm screening

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1. Introduction

Stylosanthes spp. is an excellent tropical leguminous forage, accounting for one-third of the forage varieties in artificial grasslands in tropical and south subtropical regions worldwide. MAG first reported Stylo anthracnose in Brazil, a devastating disease that can cause yellowing and necrosis of forage leaves, wilting of stems and petioles, failure of

inflorescences to set seed, and even death of seedlings and entire plants in severe cases. This disease has become a major limiting factor in the production and utilization of *Stylosanthes*, leading to sharp declines or even total losses in forage and seed yields. Obtaining anthracnose-resistant varieties of *Stylosanthes* has become an urgent problem to be solved in production [1–5]. *Stylosanthes* are self-pollinating, making cross-breeding difficult. Therefore, there is an urgent need to establish rapid screening techniques and methods for anthracnose-resistant *Stylosanthes* parents and varieties.

Jiang Changshun et al. have identified the reactions of 42 *Stylosanthes* germplasms to different anthracnose strains and screened out the highly disease-resistant Mineirao *Stylosanthes* [5–7]. At the same time, using secondary metabolite quantification techniques, they compared the secondary metabolites of susceptible Graham *Stylosanthes* and disease-resistant Mineirao *Stylosanthes*. The structures of 24 compounds were identified by spectroscopy, and two new compounds were discovered. These 24 compounds are salicylic acid (1), medicarpin (2), aglycone of crotonionoside F (3), schensiand A (4), pinoresinol (5), 3'-methoxycoumestrol (6), stigmast-5-ene-3β,7α-diol (7), linoleic acid glyceride (8), betulinic acid (9), 3-methoxykaempferol (10), (+)-3-hydroxy-β-ionone (11), soyasapogenol B (12), indazole (13), Styleguol (14), corchoionol C (15), β-daucosterol (16), caffeic acid methyl ester (17), Stylegu A (18), benzyl-2-O-β-D-glucopyranosyl-2,6-dihydroxybenzoate (19), (+)-isolariciresinol (20), vomifoliol (21), (+)-dehydrovomifoliol (22), 5''-Isopropenyldihydrofuran-(2'',3'':7,8)-5, 4'-dihydroxy dihydroflavonol (23), and β-sitosterol (24). Among the 21 compounds tested for activity against *Stylosanthes Colletotrichum gloeosporioides*, 9 compounds (1, 2, 8, 12, 13, 14, 15, 17, and 18) showed certain inhibitory zones. The antifungal activity was compared by calculating the ratio of the inhibitory zone diameter of each compound to that of the positive control carbendazim. The results showed that compound 15 had the best activity, followed by compounds 1 and 14. Compounds 12 and 13 had similar activity, while compounds 2, 8, and 17 had similar activity. Compound 18 had the least activity, but all compounds had less activity than the positive control carbendazim. The research results indicate that norsesquiterpenoids such as corchoionol C and salicylic acid in *Stylosanthes* leaves are the main factors resisting anthracnose [8–13].

This study measured and compared the contents of norsesquiterpenoids such as corchoionol C, salicylic acid, indazole, and triethyl orthobenzoate in *Stylosanthes* leaves. The aim is to establish rapid screening techniques and methods for anthracnose-resistant *Stylosanthes* parents and varieties, which will have important application value for selecting anthracnose-resistant *Stylosanthes* germplasm and breeding anthracnose-resistant *Stylosanthes* varieties.

2. Experimental materials and methods

2.1. Instruments and equipment used in the experiment

Waters ACQUITY H UPLC® CLASS ultra-high performance liquid chromatogram (with diode array detector, scanning wavelength range: 190 nm~800 nm); Centrifuge 5804 R high-speed centrifuge (Eppendorf company); Multi-tube vortex mixer (Heidolph company, Germany); Mettler XS205 analytical balance with an accuracy of 0.00001 g (Mettler Toledo company, USA); SCIENTZ-950E ultrasonic extractor (Ningbo Xinzhi Biotechnology Co., Ltd.), Rotary evaporator (Heidolph company, Germany).

2.2. Reagents used in the experiment

Acetonitrile, methanol, ethyl acetate, n-hexane, ethanol (Merck company, Germany); Soyasapogenol B, indazole, triethyl orthobenzoate, salicylic acid (First Standard company).

2.3. Plant materials

105 samples of *Stylosanthes* leaves were collected from the experimental base of the Tropical Forage Research Center of the Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences (**Table 1**).

Table 1. 105 samples of *Stylosanthes* germplasm

Serial number	Code	Chinese name	Scientific name	*Colletotrichum stylosandrae* pathogen response	Remark
1	TF 332	Reyan 25 *Stylosanthes*	*S. guianensis* cv. Reyan No.25	Resistant*	Highly resistant, high yield, cold-resistant Mineriao
2	TF 292	Reyan 5 *Stylosanthes*	*S. guianensis* cv. Reyan No.5	Susceptible*	Susceptible
3	TF 298	*Stylosanthes* graminosa	*S. guianensis cv. Graham*	Susceptible*	Highly susceptible
4	TF 184	USF 873015 Stylo	*S. guianensis* USF 873015	Susceptible*	Susceptible, brown
5	TF 180	*Stylosanthes* graminosa	*S. guianensis cv. Graham*	Susceptible*	Susceptible
6	TF 302	Reyan 10 *Stylosanthes*	*S. guianensis* cv. Reyan No.10	Susceptible*	Susceptible
7	TF 29	CPI 18750 (A) *Stylosanthes*	*S. guianensis* CPI 18750 (A)	Resistant*	Disease resistant, cold resistant
8	TF 378	USF 873015 Stylo	*S. guianensis US F873015*	Susceptible*	Susceptible, yellowish brown, cold-resistant
9	TF 380	USF 873016-1 Stylo	*S. guianensis US F873016-1*	Susceptible*	Disease susceptible, Guangdong species, cold resistant
10	TF 284	Cook Stylo	*S. guianensis* cv. Cook	Susceptible*	Infected hairy, South 02159/cook
11	TF 304	Reyan 7 *Stylosanthes*	*S. guianensis* cv. Reyan No.7	Resistant*	Disease resistance, CIAT136
12	TF 255	CIAT 1890 Stylo	*S. guianensis CIAT 1890*	Resistant*	Susceptible, few lesions
13	TF 280	CIAT 136 Stylo	*S. guianensis CIAT 1 36*	Resistant*	Disease resistance
14	TF 136	CIAT 1044 Stylo	*S. guianensis CIAT 1 044*	Resistant*	Disease resistance
15	TF 278	CIAT 1283 *Stylosanthes*	*S. guianensis CIAT 1283*	Resistant*	Disease resistance
16	TF 264	907 Stylo	*S. guianensis cv. 907*	Susceptible*	Susceptible, Black, CU 907
17	TF 291	Reyan 2 *Stylosanthes*	*S. guianensis* cv. Reyan No.2	Susceptible*	Susceptible
18	TF 276	CIAT 11371 *Stylosanthes*	*S. guianensis CIAT 11371*	Susceptible*	Susceptible, hairless
19	TF 281	CIAT 2340 *Stylosanthes*	*S. guianensis CIAT 2340*	Susceptible*	Disease resistance
20	TF 282	CIAT 2950 Stylo	*S. guianensis CIAT 2950*	Resistant*	Susceptible, hairless
21	TF 363	CIAT 11363 *Stylosanthes*	*S. guianensis CIAT 11363*	Susceptible*	Susceptible
22	TF 305	Reyan 2 *Stylosanthes*	*S. guianensis* cv. Reyan No.2	Susceptible*	Susceptible, S. guianensis Sw. “Graham”
23	TF 298	*Stylosanthes* graminosa	*S. guianensis cv. Graham*	Susceptible*	Susceptible, long leaves
24	TF 172	CIAT 184 Stylo	*S. guianensis CIAT 184*	Susceptible*	Disease-susceptible, disease-resistant (old)
25	TF 306	USF 873017 Stylo	*S. guianensis US F873017*	Susceptible*	Susceptible, E3/873017

Table 1 (Continued)

Serial number	Code	Chinese name	Scientific name	*Colletotrichum stylosandrae* pathogen response	Remark
26	TF 179	TPRC90087 Stylo	*S. guianensis* TPRC 90087	Resistant #	Good growth
27	TF 186	Nina Stylo	*S. guianensis* Nina	Resistant #	Good growth
28	TF 178	E5 Stylo	*S. guianensis* E5	Resistant #	Good growth
29	TF 189	ATF 541 Stylo	*S. guianensis* ATF 541	Resistant #	Black, good growth
30	TF 202	TPRC *Stylosanthes*	*S. guianensis* TPRC	Resistant #	Good growth
31	TF 270	South 02145/184 Stylo	S. *guianensis Nan 02145/184*	Susceptible*	Disease
32	TF 269	CIAT 184 (01) *Stylosanthes*	*S. guianensis CIAT 184*	Susceptible*	Susceptible
33	TF 25	CIAT 11128 *Stylosanthes*	*S. guianensis CIAT 11128*		*Stylosanthes scabra*
34	TF 151	CIAT11900 *Stylosanthes*	*S. guianensis CIAT 11900*	Resistant#	Highly resistant, upright *Stylosanthes*, cold-resistant and disease-resistant
35	TF 141	CIAT 10301 *Stylosanthes*	*S. guianensis CIAT 10301*	Resistant#	Medium resistance, coarse *Stylosanthes*
36	TF 273	CIAT SK *Stylosanthes*	*S. guianensis CIAT SK*	Resistant#	Medium resistance
37	TF 7	CIAT 1278 Stylo	*S. guianensis CIAT 1278*	Resistant#	Medium resistance, stylophora
38	TF 177	TPRC 90064 Stylo	*S. guianensis* TPRC 90064	Resistant#	Medium resistance, hairy/C001
39	TF 189	ATF 541 Stylo	*S. guianensis* ATF 541	Resistant#	High resistance, black
40	TF 251	CIAT 50 Stylo	*S. guianensis CIAT 50*	Resistant#	Medium resistance
41	TF 229	CIAT 11075 *Stylosanthes*	*S. guianensis CIAT 11075*	Susceptible#	Medium resistance
42	FT 226	CIAT 10598 *Stylosanthes*	*S. guianensis CIAT 10598*		
43	TF 374	L7 Stylo	*S. guianensis* L7	Susceptible#	Susceptible, hairy
44	TF 250	CIAT 2659 Stylo	*S. guianensis CIAT 2659*		
45	TF 171	CIAT 184 Stylo	*S. guianensis CIAT 184*	Susceptible*	Hairy
46	TF 352	TPRC 90028 *Stylosanthes*	*S. guianensis* TPRC 90028	Resistant#	Medium disease resistance
47	TF 257	CIAT 2439 Stylo	*S. guianensis CIAT 2439*		
48	TF 321	GC 1528/IRRI *Stylosanthes*	*S. guianensis* GC 1528	Resistant#	Medium disease resistance
49	TF 44	CIAT 2657 Stylo	*S. guianensis CIAT 2657*		*Stylophytum macrophylla*
50	TF 233	Yueyan No.1 *Stylosanthes*	*S. guianensis* cv. Yueyan No.1	Resistant#	Highly resistant, hybrid, cold-resistant and disease-resistant

Table 1 (Continued)

Serial number	Code	Chinese name	Scientific name	*Colletotrichum stylosandrae* pathogen response	Remark
51	TF 241	CIAT 149 Stylo	*S. guianensis CIAT 149*		Cold resistant
52	TF 42	CIAT 11856 *Stylosanthes*	*S. guianensis CIAT 11856*	Resistant#	Medium resistance, large leaves, cold and disease resistant
53	TF 313	FM 9405-6 Stylo	*S. guianensis* FM 9405-6	Resistant#	Medium resistance, cold and disease-resistant
54	TF 203	Product 109 Stylo	*S. guianensis* cv.pin109	Resistant#	High resistance
55	TF 41	CIAT 10121 *Stylosanthes*	*S. guianensis* CIAT 10121	Resistant#	Medium resistance, large leaves, cold and disease resistant
56	TF 391	Reyan 21 *Stylosanthes*	*S. guianensis* cv. Reyan No.21	Resistant#	Medium disease and cold resistance
57	TF 312	FM 07-3/IRRI *Stylosanthes*	*S. guianensis* FM 07-3/IRRI	Resistant#	Medium disease resistance, cold resistance
58	TF 254	CIAT 75 Stylo	*S. guianensis* CIAT 75	Susceptible#	Susceptible, cold-resistant
59	TF 332	Reyan 25 *Stylosanthes*	*S. guianensis* cv. Reyan No.25	Resistant#	High disease resistant, cold-resistant, high-yield Mineriao
60	TF 231	CIAT 11279 Stylo	*S. guianensis* CIAT 11279	Susceptible#	Medium disease and cold resistance
61	TF 205	CIAT 25 Stylo	*S. guianensis* CIAT 25	Susceptible#	Susceptible to disease
62	TF 243-1	CIAT 1723-1 *Stylosanthes*	*S. guianensis* CIAT 1723-1	Susceptible#	Highly sensitive to disease, hairy
63	TF 232	CIAT 1130 Stylo	*S. guianensis* CIAT 1130	Susceptible#	Low disease resistance, cold resistance
64	TF 242	CIAT 1602 Stylo	*S. guianensis* CIAT 1602	Susceptible#	Susceptible
65	TF 187	Temprano Stylo	*S. guianensis* Temprano	Susceptible#	Low disease resistance
66	TF 223	CIAT 10530 Stylo	*S. guianensis* CIAT 10530	Susceptible#	Susceptible to disease
67	TF 236	CIAT 1200 Stylo	*S. guianensis* CIAT 1200	Susceptible#	Medium disease resistance
68	TF 261	CIAT 67562 *Stylosanthes*	*S. guianensis* CIAT 67562	Susceptible#	Susceptible, hairy, disease-prone
69	TF 225	CIAT 10594 *Stylosanthes*	*S. guianensis* CIAT 10594	Susceptible#	Susceptible
70	TF 221	CIAT 10500 Stylo	*S. guianensis* CIAT 10500	Susceptible#	Medium disease and cold resistance
71	TF 298	*Stylosanthes* graminosa	*S. guianensis cv. Graham*	Susceptible*	Medium disease resistance
72	TF 259	CIAT 2813 *Stylosanthes*	*S. guianensis* CIAT 2813		Not cold resistant
73	TF 275	CIAT 11364 *Stylosanthes*	*S. guianensis* CIAT 11364	Resistant#	Medium disease resistance
74	TF 260	CIAT 58719 *Stylosanthes*	*S. guianensis* CIAT 58719	Resistant#	Medium disease resistance
75	TF 215	CIAT 10235 *Stylosanthes*	*S. guianensis* CIAT 10235	Resistant#	Medium disease resistance

Table 1 (Continued)

Serial number	Code	Chinese name	Scientific name	*Colletotrichum stylosandrae* pathogen response	Remark
76	TF 222	CIAT 10529 Stylo	*S. guianensis* CIAT 10529	Resistant#	Medium disease and cold resistance
77	TF 213	CIAT 11376 *Stylosanthes*	*S. guianensis* CIAT 11376	Resistant#	Medium disease resistance
78	TF 207	CIAT 151 Stylo	*S. guianensis* CIAT 151	Susceptible#	Moderate disease resistance, not cold tolerant
79	TF 246	CIAT 1948 Stylo	*S. guianensis* CIAT 1948	Resistant#	Medium disease resistance
80	TF 237	CIAT 1249 Stylo	*S. guianensis* CIAT 1249	Susceptible#	Medium susceptibility
81	TF 245	CIAT 1799 Stylo	*S. guianensis* CIAT 1799	Resistant#	Medium disease resistance
82	TF 196	South 02152 Stylo	*S. guianensis Nan* 02152	Resistant#	Medium disease resistance
83	TF 185	CIAT 11371 *Stylosanthes*	*S. guianensis* CIAT 11371	Resistant#	Medium disease resistance, unusual, hairless
84	TF 220	CIAT 10392 *Stylosanthes*	*S. guianensis* CIAT 10392	Resistant#	Medium disease resistance
85	TF 193	South 01090 Stylo	*S. guianensis Nan* 01090	Susceptible#	Medium susceptibility
86	TF 206	CIAT 104 Stylo	*S. guianensis* CIAT 104	Resistant#	Medium disease resistance
87	TF 197	South 02153 Stylo	*S. guianensis Nan* 02153	Susceptible#	Medium susceptibility
88	TF 294	Cook Stylo	*S. guianensis* cv. cook	Susceptible#	High Sensitivity
89	TF 209	CIAT 41 Stylo	*S. guianensis* CIAT 41	Susceptible#	Medium susceptibility
90	TF 266	ATF 3309 Stylo	*S. guianensis* ATF 3309	Resistant#	Medium disease resistance
91	TF 219	CIAT 10390 Stylo	*S. guianensis* CIAT 10390	Susceptible#	Medium susceptibility
92	TF 0244	CIAT 178 Stylo	*S. guianensis* CIAT 178	Resistant#	Medium disease resistance
93	TF 191	South 01084 Stylo	*S. guianensis Nan 01084*	Susceptible#	Medium susceptibility
94	TF 277	CIAT 1281 *Stylosanthes*	*S. guianensis* CIAT 1281	Resistant#	Medium disease resistance
95	TF 265	ATF 3308 Stylo	*S. guianensis* ATF 3308	Resistant#	Medium disease resistance
96	TF 262	CIAT 87830 Stylo	*S. guianensis* CIAT 87830	Resistant#	Medium disease resistance
97	TF 293	CV.scholfiold Stylo	*S. guianensis* cv. scholfiold	Resistant#	Medium disease resistance
98	TF 253	CIAT 74 Stylo	*S. guianensis* CIAT 74	Susceptible#	Medium susceptibility
99	TF 314	FM 9405-3 Stylo	*S. guianensis* FM 9405-3	Resistant#	Medium disease resistance
100	TF 279	Tandio Stylo	*S. guianensis* Tandio	Susceptible#	Medium susceptibility

Table 1 (Continued)

Serial number	Code	Chinese name	Scientific name	*Colletotrichum stylosandrae* pathogen response	Remark
101	TF 297	cv. Endeavour Stylo	*S. guianensis* cv. Endeavour	Resistant#	Medium disease resistance
102	TF 235	CIAT 11809 Stylo	*S. guianensis* CIAT 11809	Resistant#	Medium disease resistance
103	TF 331	FM 9405 Stylo	*S. guianensis* FM 9405	Resistant#	
104	TF 328	Reyan 18 *Stylosanthes*	*S. guianensis* cv. Reyan No.18	Resistant#	Medium disease resistance
105	TF 32	Brazilian Alfalfa	*S. guianensis Sw; S. gracilis H·B·K*	Susceptible#	Susceptible

Note: * indicates the results of potted plant inoculation experiments; # indicates the results of field plant observations; disease resistance is classified as low resistance, medium resistance, and high resistance; disease susceptibility is classified as susceptible, moderately susceptible, and highly susceptible

2.4. Methods

2.4.1. Preparation of standard working solution

Accurately weigh appropriate amounts of soyasapogenol B, indazole, triethyl orthobenzoate, and salicylic acid standards. Prepare a 1.00 mg/mL standard stock solution using methanol. Take 2.5 mL of the soyasapogenol B standard stock solution and 0.5 mL of each of the other three secondary metabolite standard stock solutions. Dilute to 5 mL with methanol to prepare a mixed standard solution containing 500 μg/mL of soyasapogenol B and 100 μg/mL of the other three secondary metabolites.

2.4.2. Sample pretreatment

Precisely weigh 2.0 g of the sample (for *Stylosanthes*, take the top 1–5 leaves of the new shoot, ground with liquid nitrogen) into a 50 mL centrifuge tube [14]. Add 20 mL of ethyl acetate, vortex mix for 10 minutes, sonicate for 25 minutes, and centrifuge at 9000 r/min for 5 minutes. Transfer the supernatant to another 50 mL centrifuge tube. Repeat the extraction process with 20 mL of ethyl acetate, combine the supernatants, add 3 g of C18 adsorbent, vortex mix for 10 minutes, and centrifuge at 9000 r/min for 5 minutes. Take the supernatant, evaporate it to near dryness in a water bath at 40℃, add 1.0 mL of mobile phase (60% ammonium acetate + 40% methanol), and sonicate for 1 minute at low temperature (with 2–4 ice packs). After filtering through a 0.22 μm organic filter membrane, it can be used as the test solution.

2.4.3. Chromatographic conditions

Chromatographic column: Luna® Omega Polar C18 (2.1×100 mm, 1.6 μm); column temperature: 35℃; injection volume: 1.5 μL; flow rate: 0.3 mL/min [15]. Photodiode array detector (PAD): 210 nm, 240 nm; mobile phase A: 20 mmol/L ammonium acetate aqueous solution, mobile phase B: methanol solution; gradient elution program: 0~5.00 min, 40%B; 5.00~5.01 min, 40%B~98%B; 5.01~6.00 min, 98%B; 6.00~6.01 min, 98%B~40%B; 6.01~8.00 min, 40%B.

3. Results and analysis

3.1. Establishment of a method for simultaneous analysis of multiple secondary metabolites in Stylosanthes using ultra-high performance liquid chromatography

A method using ultra-high performance liquid chromatography (UPLC) has been established to determine the content of salicylic acid, triethyl orthobenzoate, indazole, and soyasapogenol B in *Stylosanthes* (**Figure 1**). The samples were extracted with ethyl acetate, purified with C18 solid-phase adsorbent, and separated using a Luna Omega C18 (2.1×100 mm, 1.6 μm) chromatographic column. The mobile phase consisted of 20 mmol/L ammonium acetate aqueous solution/methanol with gradient elution at a flow rate of 0.3 mL/min. The detection wavelengths were 210 nm and 240 nm, the column temperature was 35℃, and the injection volume was 1.5 μL. Under these chromatographic conditions, all four secondary metabolites showed good linearity. The linear range for soyasapogenol B was 5.0~500 μg/mL, and for the other three secondary metabolites, it was 1.0~100 μg/mL. The correlation coefficient r was ≥0.996. The limits of detection for salicylic acid, triethyl orthobenzoate, indazole, and soyasapogenol B were 0.01, 0.01, 0.02, and 0.3 mg/kg, respectively, and the limits of quantitation were 0.5, 0.5, 0.5, and 2 mg/kg, respectively. At three different concentration levels, the average recovery rates ranged from 60.6% to 118.5%, the relative standard deviation (RSD) of the method was 1.1%~9.1%, and the precision RSD of the instrument (n = 6) was 0.2%~3.6%. 105 samples were tested and analyzed (**Table 2**). Among them, 26 samples

had a salicylic acid content of ≥80 μg/g, with the top five being FM 9405-6 *Stylosanthes* (157.9 μg/g), CIAT 10500 *Stylosanthes* (114.7 μg/g), CIAT 11279 *Stylosanthes* (111.8 μg/g), and CIAT 1948 *Stylosanthes* (109.4 μg/g). There were 62 samples with a total content of salicylic acid, triethyl orthobenzoate, indazole, and soyasapogenol B ≥100 μg/g. The top five were Pin 109 *Stylosanthes* (264.5 μg/g), CIAT 10301 *Stylosanthes* (258.3 μg/g), FM 9405-6 *Stylosanthes* (254.6 μg/g), cv. Scholfield *Stylosanthes* (216.7 μg/g), and CIAT 2813 *Stylosanthes* (215.2 μg/g). Among them, Mineriao *Stylosanthes* had salicylic acid (92.4 μg/g), triethyl orthobenzoate (4.4 μg/g), indazole (4.6 μg/g), and soyasapogenol B (4.8 μg/g), with a total content of 106.2 μg/g for the four secondary metabolites. Since this variety is highly resistant and high-yielding, it was used as a reference to evaluate the test results of 104 germplasm samples. The study showed that the established detection method is simple, accurate, and reproducible, and it has good application prospects for batch determination of secondary metabolite content in *Stylosanthes*.

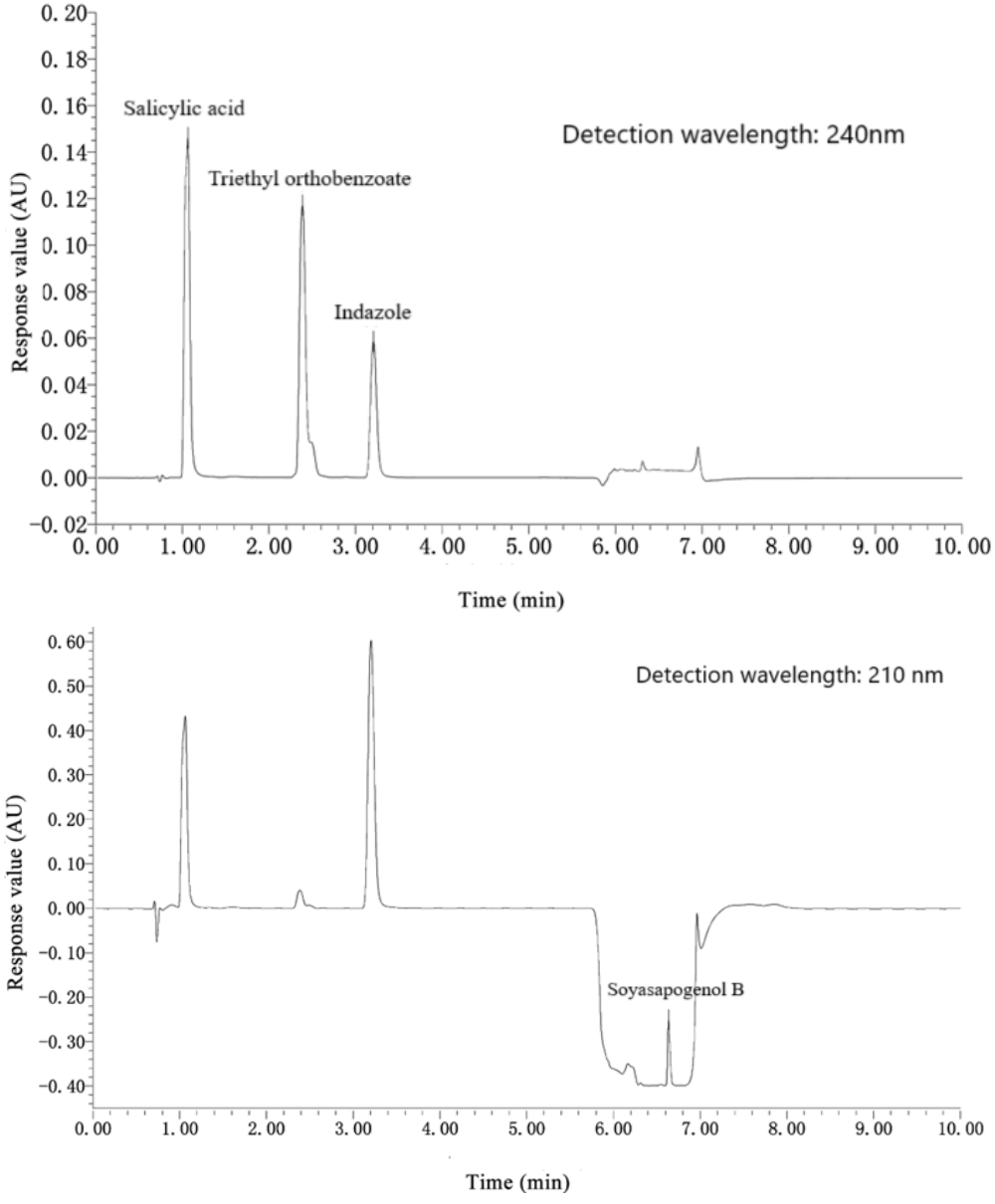

Figure 1. Chromatogram of four secondary metabolite standards

Table 2. Contents of four secondary metabolites in 105 *Stylosanthes* germplasms

Serial number	Code	Introduction name	Approved name	Anthracnose resistance	Salicylic acid μg/g	Soybean sterol B μg/g	Indazole μg/g	Corchoionol C μg/g	Total content μg/g
1	TF 332	Mineriao	Reyan 25 *Stylosanthes*	High resistance	92.4	4.8	4.6	4.4	106.2
2	TF 292		Reyan 5 *Stylosanthes*		48.3	5	1.1	3.6	58
3	TF 298	*Stylosanthes* graminosa			45.2	5.5	0.9	2.6	54.2
4	TF 184	USF 873015 Stylo			55.5	4.8	1.1	7.6	69
5	TF 180	*Stylosanthes* graminosa			42.5	4.7	0.6	3.3	51.1
6	TF 302		Reyan 10 *Stylosanthes*		44.4	4.7	0.8	2.3	52.2
7	TF 29	CPI 18750 (A) *Stylosanthes*			/	/	/	/	/
8	TF 378	USF 873015 Stylo			40.2	10.1	2.1	4.7	57.1
9	TF 380	USF 873016-1 Stylo			44.7	4.7	0.7	2.9	53
10	TF 284	Cook Stylo		Susceptible	63.8	10.2	1.6	3	78.6
11	TF 304		Reyan 7 *Stylosanthes*	Susceptible	63.1	10.2	0.9	3.9	78.1
12	TF 255	CIAT 1890 Stylo	/	Disease resistance	47.8	10.1	7.0	4.5	69.4
13	TF 280	CIAT 136 Stylo	/	Susceptible	20.3	10.2	1.0	1.4	32.9
14	TF 136	CIAT 1044 Stylo	/	Disease resistance	41.2	10.2	1.6	9.5	62.5
15	TF 278	CIAT 1283 *Stylosanthes*	/	Disease resistance	67.5	10.1	0.9	1.9	80.4
16	TF 264	907 Stylo	/	Disease resistance	49.3	10.1	0.9	2.0	62.3
17	TF 291		Reyan 2 *Stylosanthes*	Susceptible	49.0	5.4	0.9	3.6	58.9
18	TF 276	CIAT 11371 *Stylosanthes*		Susceptible	46.6	10.1	6.7	8.2	71.6
19	TF 281	CIAT 2340 *Stylosanthes*		Susceptible	59.8	10.1	2.5	3.5	75.9
20	TF 282	CIAT 2950 Stylo		Disease resistance	48.3	5.4	2.0	4.0	59.7
21	TF 363	CIAT 11363 *Stylosanthes*		Susceptible	50.3	10.1	2.1	2.8	65.3
22	TF 305		Reyan 2 *Stylosanthes*	Susceptible	50.5	5.0	0.7	4.9	61.1
23	TF 298	*Stylosanthes* graminosa		Susceptible	45.2	5.5	0.9	2.6	54.2
24	TF 172	CIAT 184 Stylo		Susceptible	41.8	10.7	0.6	1.6	54.7
25	TF 306	USF 873017 Stylo		Susceptible	33	10.5	0.8	2.2	46.5

Table 2 (Continued)

Serial number	Code	Introduction name	Approved name	Anthracnose resistance	Salicylic acid μg/g	Soybean sterol B μg/g	Indazole μg/g	Corchoionol C μg/g	Total content μg/g
26	TF 179	TPRC90087 Stylo			97.6	5.5	1.9	4.4	109.4
27	TF 186	Nina Stylo			66.5	10.3	2.7	3.2	82.7
28	TF 178	E5 Stylo			43.1	10.1	2.2	5.7	61.1
29	TF 189	ATF 541 Stylo			50.4	5.5	1.3	3.2	60.4
30	TF 202	TPRC *Stylosanthes*			45.8	10.1	0.8	1.1	57.8
31	TF 270	South 02145/184 Stylo		Susceptible	35.0	10.3	1.6	1.4	48.3
32	TF 269	CIAT 184 (01) *Stylosanthes*		Susceptible	36.3	10.1	1.9	2.2	50.5
33	TF 25	CIAT 11128 *Stylosanthes*			65.9	8.8	6.5	4.2	85.4
34	TF 151	CIAT11900 *Stylosanthes*			82.7	8.9	7.9	6.2	105.7
35	TF 141	CIAT 10301 *Stylosanthes*			75.0	8.8	46.1	128.4	258.3
36	TF 273	CIAT SK *Stylosanthes*			89.5	9.2	32.7	4.2	135.6
37	TF 7	CIAT 1278 Stylo			55.7	9.0	10.5	6.9	82.1
38	TF 177	TPRC 90064 Stylo			51.9	9.0	58.6	4.5	124
39	TF 189	ATF 541 Stylo			60.9	9.0	46.3	4.0	120.2
40	TF 251	CIAT 50 Stylo			54.7	9.0	74.4	2.8	140.9
41	TF 229	CIAT 11075 *Stylosanthes*			75.7	9.0	33.4	3.8	121.9
42	FT 226	CIAT 10598 *Stylosanthes*			/	/	/	/	/
43	TF 374	L7 Stylo			56.1	9.0	11.3	3.6	80
44	TF 250	CIAT 2659 Stylo			40.2	9.1	9.2	2.7	61.2
45	TF 171	CIAT 184 Stylo			49.3	8.9	61.8	2.3	122.3
46	TF 352	TPRC 90028 Stylo			63.5	9.0	7.2	2.4	82.1
47	TF 257	CIAT 2439 Stylo			64.2	8.9	50.0	14.6	137.7
48	TF 321	GC 1528/IRRI *Stylosanthes*			57.7	9.6	44.2	5.6	117.1
49	TF 44	CIAT 2657 Stylo			70.9	9	24.1	3.8	107.8
50	TF 233	Yueyan No.1 *Stylosanthes*		Susceptible	3.7	9.6	1.0	4.4	18.7

Table 2 (Continued)

Serial number	Code	Introduction name	Approved name	Anthracnose resistance	Salicylic acid μg/g	Soybean sterol B μg/g	Indazole μg/g	Corchoionol C μg/g	Total content μg/g
51	TF 241	CIAT 149 Stylo			55.5	8.9	53.6	8.3	126.3
52	TF 42	CIAT 11856 *Stylosanthes*			7.5	9.9	1.0	3.5	21.9
53	TF 313	FM 9405-6 Stylo			157.9	8.9	69.9	17.9	254.6
54	TF 203	Product 109 Stylo			89.2	9.2	121.7	44.4	264.5
55	TF 41	CIAT 10121 *Stylosanthes*		Large leaves, cold-resistant and disease-resistant	58.2	9.2	16.4	13.9	97.7
56	TF 391		Reyan 21 *Stylosanthes*	Cold and disease-resistant	69.6	9.9	56.8	13.7	150
57	TF 312	FM 07-3/IRRI *Stylosanthes*		Disease susceptible, Guangdong species, cold resistant	44.7	4.7	0.7	2.9	53
58	TF 254	CIAT 75 Stylo		Cold resistant	21.1	31.3	0.9	2.5	55.8
59	TF 332		Reyan 25 *Stylosanthes*	Cold resistant	81.3	9.0	72.0	9.1	171.4
60	TF 231	CIAT 11279 Stylo		Disease-resistant, cold-resistant, high-yield	111.8	8.9	76.0	14.2	210.9
61	TF 205	CIAT 25 Stylo		Susceptible	74.8	10.0	81.1	16.5	182.4
62	TF 243-1	CIAT 1723-1 *Stylosanthes*		Hairy, susceptible	85.3	8.9	47.6	4.5	146.3
63	TF 232	CIAT 1130 Stylo		Cold resistant	82.6	10.5	63.0	12.3	168.4
64	TF 242	CIAT 1602 Stylo		Susceptible	66.0	8.8	103.9	17.8	196.5
65	TF 187	Temprano Stylo		Disease resistance	43.3	8.9	81.6	9.4	143.2
66	TF 223	CIAT 10530 Stylo		Susceptible	55.5	8.9	111.4	28.4	204.2
67	TF 236	CIAT 1200 Stylo		Susceptible	68.8	8.8	65.8	14.4	157.8
68	TF 261	CIAT 67562 *Stylosanthes*		Susceptible	58.5	8.8	31.3	4.4	103
69	TF 225	CIAT 10594 *Stylosanthes*		Susceptible	53.4	8.8	58.0	8.6	128.8
70	TF 221	CIAT 10500 Stylo		Susceptible	114.7	8.8	44.3	14.4	182.2
71	TF 298	*Stylosanthes* graminosa		High Sensitivity	45.2	5.5	0.9	2.6	54.2

Table 2 (Continued)

Serial number	Code	Introduction name	Approved name	Anthracnose resistance	Salicylic acid μg/g	Soybean sterol B μg/g	Indazole μg/g	Corchoionol C μg/g	Total content μg/g
72	TF 259	CIAT 2813 *Stylosanthes*			78.4	8.8	116.1	11.9	215.2
73	TF 275	CIAT 11364 *Stylosanthes*			64.9	8.8	38.5	2.9	115.1
74	TF 260	CIAT 58719 *Stylosanthes*			81.2	8.8	52.0	3.9	145.9
75	TF 215	CIAT 10235 *Stylosanthes*			58.0	8.8	59.4	9.9	136.1
76	TF 222	CIAT 10529 Stylo			47.1	9.8	56.2	7.0	120.1
77	TF 213	CIAT 11376 *Stylosanthes*			83.5	9.2	28.0	4.9	125.6
78	TF 207	CIAT 151 Stylo			70.7	8.9	51.6	18.6	149.8
79	TF 246	CIAT 1948 Stylo			109.4	8.8	53.2	11.8	183.2
80	TF 237	CIAT 1249 Stylo			67.6	8.8	84.5	23.0	183.9
81	TF 245	CIAT 1799 Stylo			100.4	9.9	81.5	3.5	195.3
82	TF 196	South 02152 Stylo			102.0	8.8	31.6	8.7	151.1
83	TF 185	CIAT 11371 *Stylosanthes*			80.6	8.8	22.3	3.3	115
84	TF 220	CIAT 10392 *Stylosanthes*			61.4	8.8	26.6	8.0	104.8
85	TF 193	South 01090 Stylo			67.5	9.1	60.3	13.3	150.2
86	TF 206	CIAT 104 Stylo			50.1	31.9	89.8	17.5	189.3
87	TF 197	South 02153 Stylo			115.4	30.1	52.0	15.7	213.2
88	TF 294	Cook Stylo		High Sensitivity	42.8	11.0	69.5	7.2	130.5
89	TF 209	CIAT 41 Stylo			69.6	8.7	71.6	19.3	169.2
90	TF 266	ATF 3309 Stylo			85.2	8.6	40.1	8.0	141.9
91	TF 219	CIAT 10390 Stylo			93.2	8.7	94.4	13.9	210.2
92	TF 0244	CIAT 178 Stylo			79.5	8.6	71.9	27.3	187.3
93	TF 191	South 01084 Stylo			54.8	8.7	31.9	2.1	97.5
94	TF 277	CIAT 1281 *Stylosanthes*			60.5	8.6	57.1	11.4	137.6
95	TF 265	ATF 3308 Stylo			84.4	8.6	53.2	10.1	156.3
96	TF 262	CIAT 87830 Stylo			63.8	9.8	40.9	4.0	118.5

Table 2 (Continued)

Serial number	Code	Introduction name	Approved name	Anthracnose resistance	Salicylic acid μg/g	Soybean sterol B μg/g	Indazole μg/g	Corchoionol C μg/g	Total content μg/g
97	TF 293	CV.scholfiold Stylo			88.1	9.2	104.0	15.4	216.7
98	TF 253	CIAT 74 Stylo			46.8	8.8	52.9	14.6	123.1
99	TF 314	FM 9405-3 Stylo			99.5	9.4	37.2	3.7	149.8
100	TF 279	Tandio Stylo			61.7	8.6	35.9	6.6	112.8
101	TF 297	cv. Endeavour Stylo			84.0	10.2	61.6	7.9	163.7
102	TF 235	CIAT 11809 Stylo			65.4	8.6	68.6	12.6	155.2
103	TF 331	FM 9405 Stylo			102.1	8.7	61.5	14.0	186.3
104	TF 328		Reyan 18 *Stylosanthes*		88.5	8.6	32.9	4.8	134.8
105	TF 32	Brazilian Alfalfa			100.7	8.6	54.8	7.3	171.4

3.2. Relationship between secondary metabolites and resistance to anthracnose in Stylosanthes

Through the analysis of 105 accessions of *Stylosanthes* and resistance comparison studies, the relationship between four secondary metabolites and disease resistance was analyzed, showing that salicylic acid is the most important, followed by indazole, triethyl benzoate, and then stigmasterol B. The total content of the four secondary metabolites is also an important indicator of *Stylosanthes*' resistance to anthracnose. The relationship diagrams of the four secondary metabolites against disease resistance are as follows (**Figure 1–5**).

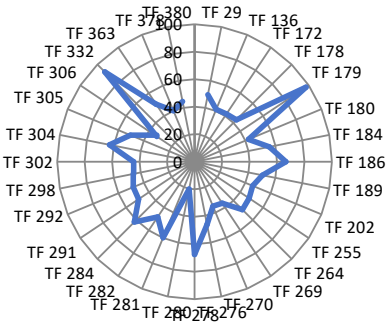

Figure 1. Relationship between salicylic acid content and resistance of *Stylosanthes* parents and resistant varieties

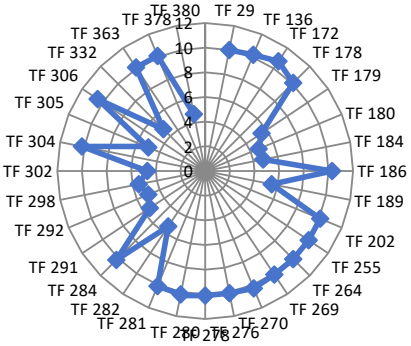

Figure 2. Relationship between Stigmasterol B content and resistance of *Stylosanthes* parents and resistant varieties

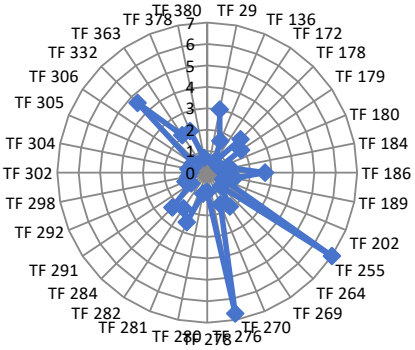

Figure 3. Relationship between indazole content and resistance of *Stylosanthes* parents and resistant varieties

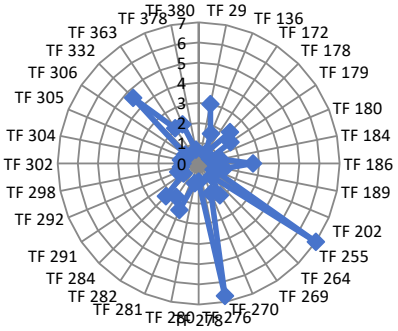

Figure 4. Relationship between triethyl benzoate content and resistance of *Stylosanthes* parents and resistant varieties

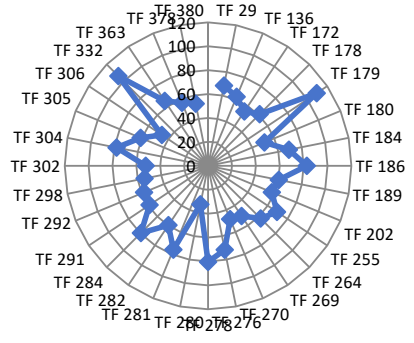

Figure 5. Relationship between the total content of four secondary metabolites and the resistance of *Stylosanthes* parents and resistant varieties

3.3. A rapid method for identifying resistant germplasm based on salicylic acid content in Stylosanthes has been established

For example, the disease-resistant *Stylosanthes* species Mineirao has a high salicylic acid content of 92.4 μg/g, while the highly susceptible species Graham and Cook have lower salicylic acid contents of 45.2 μg/g and 42.8 μg/g, respectively. The specific steps of the method are as follows. Step 1: Collect 8 g to 10 g of leaves from the first to fifth whorls of new shoots of *Stylosanthes* (the best sampling time is from 8:00 to 10:00 am in June to August). Step 2: Preprocess the samples. Grind the samples with liquid nitrogen within 24 hours of collection and store them at -20℃. Step 3: Extract the four secondary metabolites from the samples. The optimal extraction conditions are a solid-liquid ratio of 2:40 (g/mL), an ultrasonic temperature of 40℃, an ultrasonic time of 30 minutes, and a solid phase adsorbent amount of 3.0 g. Step 4: Determine the content of the four secondary metabolites in the samples. Method used: UPLC method to determine the content of four secondary metabolites in *Stylosanthes*. The optimal detection conditions are separation using a Luna Omega C18 (2.1×100 mm, 1.6 μm) chromatographic column, with 20 mmol/L ammonium acetate aqueous solution/methanol as the mobile phase, gradient elution, a flow rate of 0.3 mL/min, detection wavelengths of 210 nm and 240 nm, a column temperature of 35℃, and an injection volume of 1.5 μL. Under these chromatographic conditions, all four secondary metabolites show good linearity. The linear range of stigmasterol B is 5.0 to 500 μg/mL, and the linear range of the other three secondary metabolites is 1.0 to 100 μg/mL. The correlation coefficient r is ≥0.996. The detection limits of salicylic acid, triethyl benzoate, indazole, and stigmasterol B are 0.01, 0.01, 0.02, and 0.3 mg/kg, respectively, and the quantification limits are 0.5, 0.5, 0.5, and 2 mg/kg, respectively. Equipment used: Waters ACQUITY H UPLC® CLASS ultra-high-performance liquid chromatograph (with diode array detector, scanning wavelength range: 190 nm to 800 nm), Centrifuge 5804 R high-speed centrifuge (Eppendorf company); Multi-tube vortex mixer (Heidolph, Germany), Mettler XS205 analytical balance (Mettler Toledo, USA), SCIENTZ-950E ultrasonic extractor (Ningbo Scientz Biotechnology Co., Ltd.), and rotary evaporator (Heidolph, Germany). Step 5: Rapid screening technology and method for resistant parents and varieties of *Stylosanthes* against anthracnose Based on the test results, evaluate and screen according to the evaluation table of anthracnose-resistant *Stylosanthes* germplasm (**Table 3**).

Table 3. Evaluation table of anthracnose-resistant *Stylosanthes* germplasm

Secondary metabolites name	Evaluation index (μg/g)						Remark
	High resistance	Medium resistance	Low resistance	Susceptible	Medium susceptibility	High susceptibility	
Salicylic acid	≥80	70–80	60–70	50–60	40–50	≤40	Important indicators
Triethyl orthobenzoate	≥4	3–4	2–3	2–3	2–3	≤2	Reference indicators
Indazole	≥4	3–4	2–3	1–2	1–2	≤1	Reference indicators
Soybean sterol B	≥10	5–10	5–10	5–10	5–10	≤5	Reference indicators
Sum	≥100	80–100	60–80	40–60	20–40	≤20	Important indicators

Step 6: Comprehensive evaluation of disease resistance of germplasm based on cultivation resistance performance.

3.4. Screening and obtaining a batch of Stylosanthes germplasm resistant to anthracnose

Using the above methods, five highly anthracnose-resistant accessions of *Stylosanthes*, including CIAT TPRC90087, CIAT 1283, Nina, FM 9405-6, and CIAT 10500, were screened.

4. Discussion

The rapid method established in this study for identifying resistant germplasm using salicylic acid content in *Stylosanthes* can be applied to large-scale screening of *Stylosanthes* germplasm resistant to anthracnose and identification of *Stylosanthes* varieties resistant to anthracnose. It is characterized by rapidness, time-saving, low cost, and accuracy.

The *Stylosanthes* germplasm resistant to anthracnose screened in this study through secondary metabolites can be further subjected to inoculation identification to cultivate new varieties of *Stylosanthes* resistant to anthracnose.

This study established a method to identify *Stylosanthes* resistance by measuring differences in the types and contents of antibacterial secondary metabolites, providing a theoretical foundation and facilitating accelerated breeding progress.

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Disclosure statement

The author declares no conflict of interest.

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