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Histological Aspects of Disease Resistance in Pearl Millet against *Sclerospora graminicola*

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ABSTRACT

Two cultivars of Pennisetum glaucum viz., 7042S and IP18294, highly susceptible and highly resistant to virulent pathotype 1 of S. graminicola, and a virulent pathotype 1 of S. graminicola was used in the present investigation. The commonly available medicinal plants, viz., Cymbopogan citrates, Zingiber officinale, Trigonella corniculata, Cicca acida, Murraya koenigii were grinded with distilled water, acetone, chloroform and methanol at 4 ^{o}C and extract was used as inducer of resistance. After preliminary studies like seedling vigor, vegetative and reproductive growth parameters, selected inducer in solvent was used to induce the resistance in plants by seed treatment. In the experimental sets, the histological parameters like host tissue necrosis, quantum of lignin, tannin, suberin and phenolics were tested. The results clearly indicated that the curry leaf extract induced remarkable resistance when compared to other experimental sets. Thus the results of this study contribute to development of a promising method to effectively inoculate the seeds with required inducer of resistance.

Key words: *Pearl millet, Sclerospora graminicola, Systemic acquired resistance.*

INTRODUCTION

Pearl Millet, *Pennisetum glaucum* is a staple food and fodder crop of semi-arid-tropics grown under erratic climatic conditions. The downy mildew disease reduces pearl millet production by about 30% to 270 million US dollars during epidemics[21]. At present, downy mildew disease has been managed by the use of cultural practices, fungicide and resistant cultivars. However, they have their own limitations. Of different methods of crop protection, one is the induction of resistance in plants against pathogens without a known alteration of genome. Recent reports clearly indicated the possibility of inducing systemic acquired resistance in host tissues against pathogens by seed treatment with systemic acquired resistance inducers[4]. Induction of resistance using various biotic agents has been studied most extensively in several plant species.

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The use of biotic factors have their own limitation such as the outbreak of disease throughout the growing season, culturing of the organism which is laborious, time consuming and is not cost effective. Recently several scientists are being investigated the possible alternatives to chemicals for plant disease management. Shetty *et al.*,[22] have tested aqueous extracts of leaves, bark, stem and Seeds of *Strychnos muxvomica*, garlic bulbs, ginger rhizomes, basil leaves and fruits of *Azadirachta indica* to control *T. paddwikii* in rice seeds. In view of the above observations, the present investigation was undertaken to study the induction of resistance and its histological aspects of disease resistance in pearl millet against *Sclerospora graminicola*.

MATERIAL AND METHODS

Host Plant: Cultivars of Pearl Millet *viz.*, 7042S and IP18294, highly susceptible and highly resistant to virulent pathotype 1 of *S. graminicola*, obtained from International Crop Research Institute for Semi- Arid Tropics, Hyderabad, India, were used for the study.

Pathogen: A virulent pathotype 1 of *S. graminicola* isolated from and maintained on the Pearl Millet cultivar (7042 S) under green house conditions was used.

Solvents: Total components of the test biotic inducers were extracted with distilled water, acetone, chloroform and methanol at 4 ^{o}C.

Biotic inducer: Biotic inducers used in the study include leaf materials of the commonly available medicinal plants, *viz*., *Cymbopogan citrates* (lemon grass), *Zingiber officinale* (Ginger), *Trigonella corniculata* (Fenugreek), *Cicca acida* (Gooseberry), *Murraya koenigii* (curry leaves). 25 grams of each sample was grinded into fine paste with test solvents at 4^{o}C. Later the samples were centrifuged at 10,000 g for 15 minutes. Later the supernatant solution was air dried to obtain fine powder. This powder was dissolved in distilled water (25 ml) and appropriate dilutions made and used as a source of inducer. Initially the test inducers were tested for their effect on germination at a concentration of 1 to 100% v/v. Those concentrations, which did not affect the germination, were selected to test their effect on the zoospore release of *S. graminicola*.

Test for antifungal activity: Antifungal activity of the test inducers were tested according to shetty *et al.*,[22].

Seed treatment with inducers and their effect on germination: Seeds of pearl millet surfaced sterilized with 0.01% sodium azide for 5 minutes followed by thorough washing in sterile distilled water to remove the traces sodium azide were immersed in different concentrations *viz.*, 10, 20, 30,40,50,60,70,80,90 and 100% inducer in distilled water (w/v) at 20^{o}C for 1-8 hours. After treatment the seeds were washed in tap water for 20-30 seconds to remove excess adhering inducer and then air dried in laminar airflow for 3-4 hours until the seeds regained their original weight. Germination tests were done according to ISTA, 2005 specification by placing the seeds between sheets of moistened paper towels at 25 ^{o}C. The seeds, which received distilled water treatment were used as controls.

Inducer treatment: Concentration of the inducer in distilled water, which did not affect the germination, was used to test for its efficacy to induce resistance in seeds of pearl millet against downy mildew disease in the present study. These treated seeds were sown onto earthen pots consisting of soil, sand and manure mix of 1:1:1 and watered regularly and maintained under green house condition. Corresponding control sets were also maintained with seeds treated in sterile distilled water.

Collection of sporangia and preparation of inoculum: Collection of sporangia and release of zoospores was as per Saffeeulla[17]. For challenge inoculation; the zoospore inoculum at a concentration of $3x10^4$ ml^{-1} was used.

Sample Preparation: The seeds were plated on moistened blotters and incubated in BOD incubator at 25$\pm$2^{o}C. For each test three replicates of 25 seeds each were considered. For histochemical analysis the seedlings were challenge inoculated with $3x10^4$ zoospores ml^{-}

[1] by root dip method according to Safeeulla[17]. Seeds that received distilled water treatment and resistant cultivars, which were subjected to germination and pathogen inoculation as above, were used as controls.

Host tissue necrosis: The three-day-old seedlings of inducer treated, untreated and resistant pearl millet seeds were inoculated with the pathogen separately. Seedlings of same age inoculated with sterile distilled water served as control. There were four replicates of 25 seedlings for each sample. The seedlings were observed at hourly intervals for a period of 24 hr for the appearance of necrotic streaks on the seedling.

Histochemical detection of Lignin: The resistant, inducer treated and untreated seedlings were decolorized in hot 70% (v/v) ethanol and soaked in hot water. Epidermal strips were scraped away from the coleoptile tissue with a scalpel blade. Phloroglucinol-HCl test for lignin[20] was carried out to test for the presence of lignin. Tissues were soaked in a solution of 2% (w/v) phloroglucinol in 95% ethanol for 1 to 2 h, then placed in a drop of 35% (v/v) hydrochloric acid on a slide and heated over low flame until the veins turned reddish purple. The slides were then observed under light microscope (Wild Leitz, Switzerland) for lignin deposition.

Histochemical detection of Tannins: The host cell death at the region of browning (necrosis) was selected for staining according to Fedar and o'brein *et al.,*[6]. The coleoptile from seedlings after 24 h of inoculation with the pathogen was used for the study. Ten seedlings from each sample were tested. A thin strip of epidermal tissue was peeled out and immersed in a solution of 0.1 % of Tryphan blue in potassium phosphate buffer (pH 7.6). After twenty min. the sample was mounted on a slide and observed under a light microscope (Wild Leitz).

Histochemical detection of Suberin: The necrotic region was selected for staining according to Faulkner and Kimmins[5]. The coleoptile from seedlings after 24 h of inoculation with the pathogen was used for the study. Ten seedlings from each sample were tested. A thin strip of epidermal tissue was peeled out and immersed in a solution of gentian violet solution. After 5 min. the sample was mounted on a slide and observed under a light microscope (Wild Leitz).

Histochemical detection of Phenolics: The necrotic region was selected for staining according to Faulkner and Kimmins[5]. The coleoptile from seedlings after 24 h of inoculation with the pathogen was used for the study. Ten seedlings from each sample were tested. A thin strip of epidermal tissue was peeled out and immersed in a solution of Prussian blue 2% in potassium phosphate buffer (pH 7.6). After 15 min. the sample was mounted on a slide and observed under a light microscope (Wild Leitz).

RESULTS AND DISCUSSION

On identifying the best concentration of the solvent extract which does not affect the germination and the duration of treatment, the seeds were treated with varying concentration of chemical inducer listed for identification of efficient resistance inducer against downy mildew. Also, the antifungal activity test concluded that the test inducers had no direct antifungal activity. Of all the biotic inducer tested, distilled water extracts of curry leaves at 40% w/v showed comparatively higher induction of resistance in pearl millet against downy mildew. Hence this inducer was further evaluated for hypersensitive reaction. The results showed hypersensitive response in seedlings of the entire three categories - induced resistant in comparison with that of a highly susceptible and highly resistant pearl millet cultivar but to varying degrees (Table 1 and Fig 1). Analysis of the time course of necrosis appearance indicated that the response was more rapid in the highly resistant seedlings with necrosis appearing as early as 30 min after inoculation. However, clear appearance in resistant cultivar was after 3 hours of inoculation. Appearance of hypersensitive reaction varying from 2 hours in inducer treated to 8 hours in untreated susceptible was observed. Tissue necrosis was observed only in coleoptiles of highly

susceptible cultivar while induced resistance and highly resistance seedlings showed necrotic coleoptiles and roots. Size and number of necrotic spots in induced resistant seedlings were more compared to those of highly susceptible seedlings.

Table 1: Time of appearance of cell necrosis in 3 day old seedlings of pearl millet after inoculation with *Sclerospora graminicola*

Treatment ↓	Number of seedlings with necrosis at different time after inoculation (h)											
	2	4	6	8	10	12	14	16	18	20	22	24
Untreated Susceptible*	_	_	_	2	2	4	5	7	20	20	20	20
Resistant*	_	10	10	16	22	24	25	25	25	25	25	25
Inducer treated Susceptible	2	3	6	13	19	22	22	23	23	23	24	24

*Control - Unprotected susceptible 7042 S Seeds treated with distilled water.

- Untreated resistant IP18294 Seeds treated with distilled water.

Inducer treated Susceptible (7042 S) treated with 40% of distilled water extract of curry leaves.

Average of two independent experiments with four replicates of 25 seedlings each.

Fig. 1: Pearl Millet seedlings treated with 40% curry leaves extract showing necrotic spots on coleoptile leaf and root

Histochemical detection of Lignin

In induced resistannce, highly susceptible and resistant seedlings, the epidermal cells in coleoptile exhibited positive reaction for lignin. Presence of lignin was indicated by the purplish red coloration of cells. Intensity of coloration indicated concentration of lignin. Observation showed that in resistant cells, lignified cells stained intense red (Fig. 2). In susceptible seedlings reddish tinge only was observed, whereas in induced resistant seedlings, coloration was intermediate between that of resistant and susceptible.

Histochemical detection of Tannins

All the test samples showed positive reaction to tryphan blue staining. Regions where tannins were present were stained green to blue. Accordingly in resistant cultivars, there

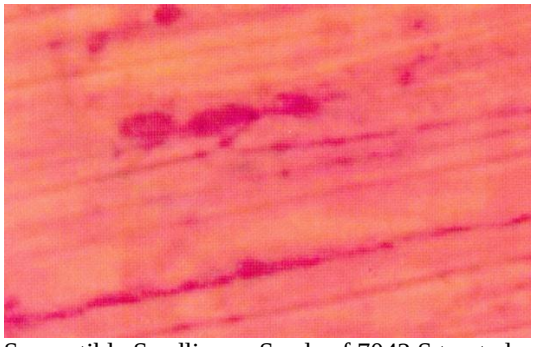
was increase in intensity when compared to the susceptible. However the induced resistant samples showed an intermediate reaction. Among the induced resistant samples, epidermal peelings of coleoptiles of pearl millet treated with distilled water extracts of curry leaves showed increased intensity compared to that of epidermal peelings of samples treated with distilled water (Fig. 3).

Histochemical detection of Suberin

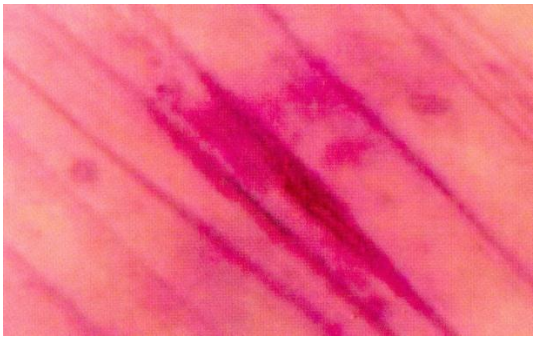
Localization of suberin was observed as pinkish coloration at the regions of its occurrence (Fig. 4). Its occurrence varied with samples tested. Maximum coloration was observed in resistant with least in susceptible cultivar of pearl millet. Among the induced resistant samples, epidermal peelings of coleoptiles of pearl millet treated with distilled water extracts of curry leaves showed increased intensity compared to that of epidermal peelings of samples treated with distilled water.

Histochemical detection of phenolics

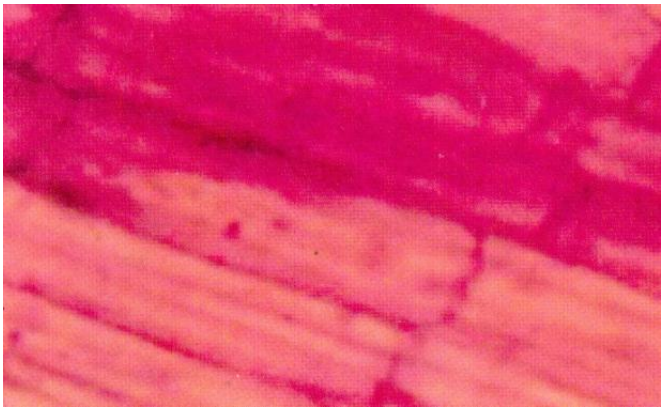
Localization of phenolics was observed as greenish coloration at the regions of its occurrence. Its deposition varied with samples tested. Maximum coloration was observed in resistant with least in susceptible cultivar of Pearl Millet (Fig 5). In induced resistant peelings, samples treated with distilled water extracts of curry leaves showed comparatively increased intensity with those treated with distilled water.



Susceptible Seedlings - Seeds of 7042 S treated with distilled water

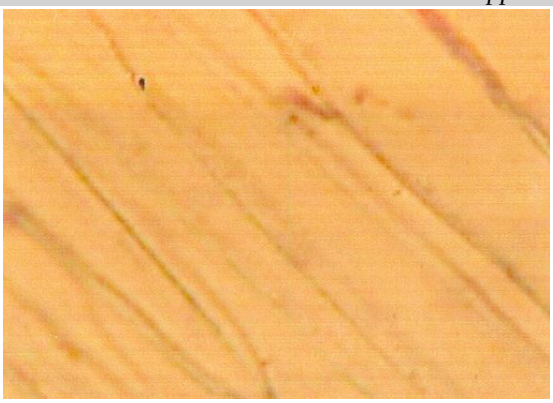


Resistant Seedlings



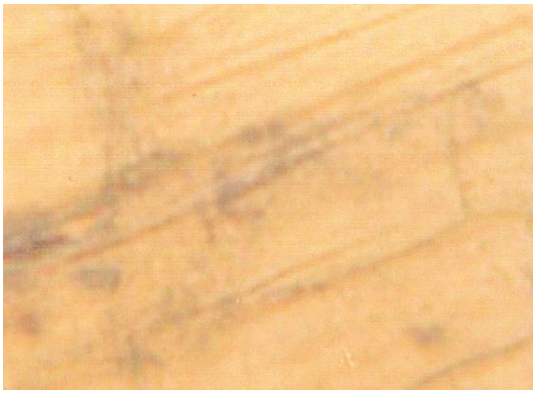
Induced Resistant Seedlings – Seeds of 7042 S treated with 40% of curry leaves extract

Fig. 2: Coleoptile epidermal peelings of pearl millet seedlings indicating lignin depositions



Susceptible Seedlings - Seeds of 7042 S treated with distilled water

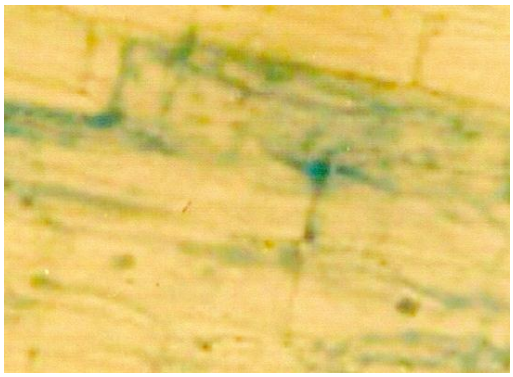
Resistant Seedlings



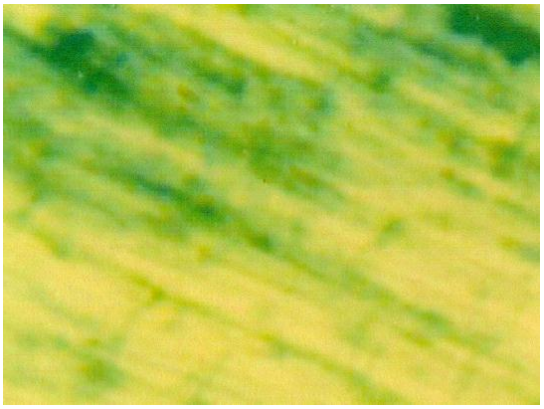
Induced Resistant Seedlings - Seeds of 7042 S treated with 40% of curry leaves extract

Fig. 3: Coleoptile epidermal peelings of pearl millet seedlings indicating tanin depositions

Susceptible Seedlings - Seeds of 7042 S treated with distilled water

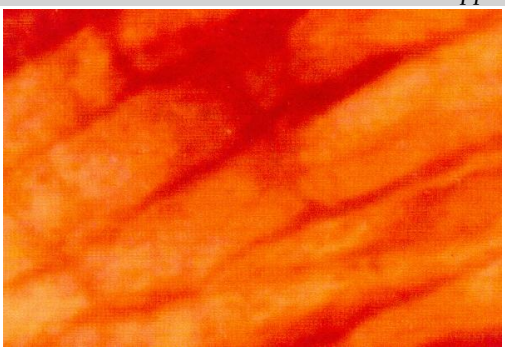


Resistant Seedlings

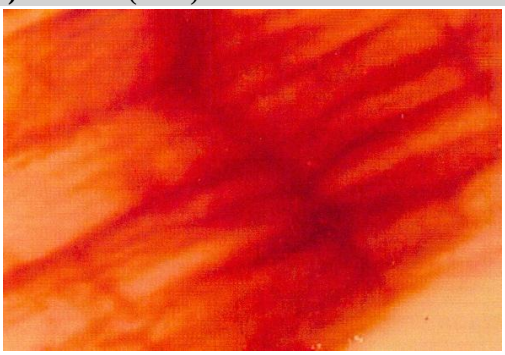


Induced Resistant Seedlings - Seeds of 7042 S treated with 40% of curry leaves extract

Fig. 4: Coleoptile epidermal peelings of pearl millet seedlings indicating suberin depositions



Susceptible Seedlings - Seeds of 7042 S treated with distilled water



Resistant Seedlings

Induced Resistant Seedlings – Seeds of 7042 S treated with 40% of curry leaves extract

Fig. 5: Coleoptile epidermal peelings of pearl millet seedlings indicating phenolics depositions

Resistance in plants is highly versatile and elastic. Even the susceptible plants can be protected by inducing resistance[4]. Induction of resistance being a technique has proved to be promising in control of downy mildew in pearl millet[7]. Though establishment of systemic acquired resistance (SAR) in seedlings on prior inoculation with sub optimal dose of the virulent pathotype of *S. graminicola*, the method was not ideal, time consuming and possibility of outbreak of disease was more. Therefore, an approach of seed treatment was developed. Seed treatment is a practical approach to fulfill all the objectives of acquired resistance and offers the advantage over other control measures for easy application under commercial agricultural conditions. Reports on induction of SAR by seed treatment with SAR inducers from plant extracts[2] are also available. However, since seed treatment with plant extracts has not been yet attempted in pearl millet, the extracts of commonly available medicinal plants were tested for their efficacy to induce resistance.

On evaluation of the seedlings for expression of downy mildew disease, it was observed that the seedlings raised from seeds that were treated with 40% of distilled water extracts of curry leaves showed significantly reduced downy mildew disease, hence more disease protection[23] in comparison with the other treatments. Pearl millet seeds treated with a solution of distilled water extracts of curry leaves exhibited significant increase in root and shoot length along with increase in germination compared to seeds soaked only in distilled water[14]. The Changes in histochemical parameters in the plant on induction of resistance were observed. Early appearance of necrotic spots in induced resistance plants than that of susceptible plants indicates the involvement of hypersensitive reaction in inducing resistance in susceptible plants.

SAR acts through fortifying the physical and mechanical strength of the cell wall as well as changing the physiological and biochemical reaction of the host leading to the synthesis of defense chemicals against the

challenge pathogen. A structural and ultrastructural cell wall modification in the host plants leads to the success of a plant in warding off invading pathogens. This relies primarily on its ability to build a line of defense rapidly for protecting cell walls against the spread of a pathogen. Seed treatment of barley seeds with plant extracts as inducers resulted in formation of structural barriers, *viz*., cell wall apposition (papillae) and deposition of newly formed callose, suberin and accumulation of phenolic compounds at the site of penetration of invading hyphae of the pathogen[24]. In cow pea, cell wall thickening, deposition of phenolic compounds and formation of pectic acids restricted the growth of *Erysiphe cichoracearum*[9]. Such a rapid defense reaction at sites of fungal entry delays the infection processes and allows sufficient time for the host to build up other defense reactions to restrict pathogen growth to the outer layers of the host tissues.

Frequency of occurrence of cell death at the site of infection is related to the downy mildew disease reaction. The highly resistant cultivar of pearl millet had more intensity of test compound than in the coleoptiles of inducer treated seedlings whereas the highly susceptible had comparatively less. This suggests that resistant cultivars check the entry of the pathogen by increasing the cell necrosis at and around infection site. Similar observation has been reported by Quiroga *et al.*,[15] and Sedlarova and Lebeda[18]. Rapid cell death leads to hypersensitive response resulting in limited spread of the pathogen. In *S. graminicola*, host-pathogen interaction, association of host necrosis with cell death and disease resistance has been reported by Sharada *et.al*.,[19] and Kumudini and shetty[11]. Differential reaction exhibited by susceptible, inducer treated and resistant pearl millet with respect to cell necrosis has been correlated to induction of resistance in our study (Table 1).

The observations made from the present study reveal that there is striking difference in the cell wall content of the highly susceptible, inducer treated and highly resistant cultivar of pearl millet following the infection with *S. graminicola.* In highly resistant, the test components considerable increased after 24 hr. of inoculation. In highly susceptible low concentration was observed when compared with that of highly resistant, where as in inducer treated, the accumulation of test compounds was intermediate. In several host-pathogen interactions[3], lignin concentrations in infected tissues have been shown to increase after infection. It is clear from the present study that lignin has a role in imparting resistance to downy mildew disease in pearl millet. Lherminier *et al*.,[12] hypothised that the presence of phenolic precursors and the production of free radicals during the process of lignification create a fungitoxic environment which adversely affects the fungus causing fungal development to cease at an early stage. *De*Cal *et al*.,[3] have observed phenolics, lignin, suberin, callose and tannins encasing invading cells of Tomato by *Fusarium oxysporum* on induction with extracts of *Penicillium oxalicum*. All these changes in structures prevent fungal growth and induce host cell death by interrupting the nutrient flow into and from host cells.

Establishment of acquired resistance to pathogens may be induced by physiological and/or developmental changes taking place in host growing plants. Indeed, the occurrence of a transition from susceptibility to resistance during development is a widely reported phenomenon in monocotyledons in the case of fungi[13, 1] and oomycetes[16, 25, 10]. On the contrary, numerous studies have investigated defense mechanisms activated in response to pathogen infection and associated to plant disease resistance[8]. These studies have underlined the key role of host-secreted molecules which accumulate in the extracellular space and contribute to the control of invasion pathogens. Synthesis and secretion of defense-related proteins are a critical part of the establishment of resistance. A defensive role of some proteins encoded by constitutively expressed genes involved mainly in metabolism but pathogenesis related genes by function are strongly suggested by their antimicrobial activity.

Thus the results of this study contribute to development of a promising method to effectively inoculate the seeds with required inducer of resistance. The technology employed utilizes activation or enhancement of plants defense mechanism. This system of resistance for protection against downy mildew is effective and suggests the possibility of returning the use of some cultivars of pearl millet which all the desired qualities but have been withdrawn from cultivation due to their susceptibility to downy mildew.

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