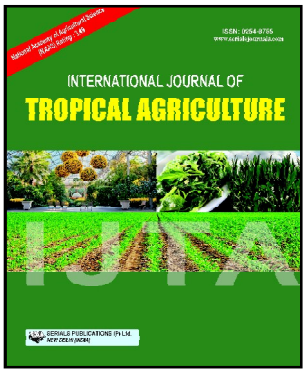


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# *In vitro* studies on *Corynespora* Leaf Fall Disease tolerance in *Hevea brasiliensis*

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***Abstract:*** *Corynespora cassiicola* is a devastating fungal pathogen which causes *Corynespora* Leaf Fall disease (CLFD) in rubber tree (*Hevea brasiliensis).* Severity of *Corynespora* infection varies among different clones of *Hevea*. Cassiicolin, the toxin secreted by the fungus, is the primary determinant of *Corynespora* pathogenicity. In the present study, susceptibility/tolerance of various clones of *Hevea* towards CLFD, as observed in field conditions, has been confirmed *in vitro*. Also, a positive correlation has been established between high chitinase activity and tolerance towards CLFD. Chitinase activity was assayed in the leaves of field grown *Hevea* clones, belonging to both susceptible and tolerant groups. Significantly higher chitinase activity was observed in tolerant clones as compared to susceptible ones. Results from these studies open up the scope of *in vitro* selection of pipeline clones tolerant towards CLFD. *In vitro* development of *Corynespora* resistant plants was attempted using callus cultures of RRII 105, the most popular and widely cultivated clone which is highly susceptible to CLFD. Embryogenic calli were cultured over medium fortified with cassiicolin. Toxin insensitive calli selected *in vitro* could be regenerated into plantlets with enhanced chitinase activity, which in turn may contribute towards tolerance to CLFD. Once confirmed, this approach can be employed for *in vitro* development of *Corynespora* resistant *Hevea* plants.

***Keywords:*** Cassiicolin, Chitinase, Corynespora Leaf Fall Disease, *Hevea brasiliensis, In vitro*

## INTRODUCTION

Brazilian rubber tree, *Hevea brasiliensis* is the most recent domesticated tree species producing Natural Rubber (NR), a product of commercial importance recovered from its latex. At present, more than 9.5 million hectares in about 40 countries are devoted to rubber tree cultivation with a production of about 6.5 million tons of dry rubber each year. The world

supply of NR is barely keeping up with a global demand for 12 million tons in 2020 [1]. Unlike most crops, rubber is comparatively little troubled by pest attacks. Among the various challenges that affect the rubber tree, fungal diseases are the most important [2]. *Corynespora* leaf fall disease (CLFD) caused by the fungus *Corynespora cassiicola* is one of the most destructive leaf diseases affecting rubber in many Asian and African countries. It affects young and old leaves of both immature and mature rubber trees. CLFD poses a serious threat to rubber cultivation since the infected leaves develop necrotic lesions and abscise, leaving the tree unproductive. Infection is made through a toxin namely cassiicolin that was discovered by Onesirosan [3], then purified and characterised by Breton [4].

*Hevea* clones clearly exhibit variable levels of susceptibility to pathogenic diseases [5]. In India, almost all clones were reported to be affected by CLFD [6]. The clone PB 260 was highly susceptible and GT1 showed resistance among the nine clones studied *in vitro* using conidial suspension inoculation method [7]. Manju [8] reported the susceptibility and distribution of CLFD in South India, according to which clone RRII 105 recorded the highest susceptibility while GT1 was the least susceptible, whereas the clones PB 217, PB 235 and PB 260 showed moderate infection to CLFD. Among RRII 400 series clones, RRII 414 and 430 were found to be comparatively less infected by the pathogen under the assistance of prophylactic fungicidal spray [9].

A basic knowledge about the biology of the causal agent and its relationship with the host plant is essential and will be highly useful for the development of suitable methods of screening and selection for resistance [10]. There is a broad range of different methodological approaches available to detect resistant genotypes and to select plants with improved resistance [11-14] among which *in vitro* screening is one of the most high-throughput and efficient methods [15]. Breton [4] suggested that the toxin can be used directly for screening the clones in the laboratory; but laboratory assessment and field observations seem to provide different rankings of the cultivated clones. It is still difficult to propose tolerant clones with a good level of confidence, and the need to choose tolerant clones strongly reduces the diversity of the clones that can possibly be used in affected areas.

Higher plants have developed a broad range of defence mechanisms against attack by fungal pathogens which include biochemical and physiological changes such as physical strengthening of cell wall through lignification, suberization and callose deposition, production of phytoalexins and synthesis of pathogenesis related (PR) proteins such as β -1,3 glucanases and chitinases [16]. Among these, production and accumulation of PR proteins in plants in response to invading pathogens and certain stress situation, is important. This is due to the immediate realization that these hydrolytic enzymes could degrade cell walls of certain fungi. The application of toxin to cultures *in vitro* can trigger the elicitation of various defence responses, e.g. phytoalexins; activity of certain enzymes [17-19]. Various studies have shown that chitinase expression against phyto-pathogen systems is higher and induction is stronger in the resistant varieties in comparison to susceptible varieties e.g. sugar beet [20], wheat [21] and tomato varieties [22].

The possibility of using toxins as selection agents to screen *in vitro* cultures of host plants is gaining popularity nowadays. Tissue culture technique facilitates the screening of many clones within a short period in a relatively small space by using crude or partially purified pathotoxin. A positive correlation has been found between toxin resistance at the cellular level and resistance to the pathogen at the plant level [23, 24].

Since Carlson [25] first successfully obtained somaclonal variants resistant to tobacco wildfire by using tissue culture techniques, *in vitro* selection strategy has been developed as an alternative for conventional breeding approach for disease

resistance. Hammerschlag [26] screened embryogenic callus derived from immature zygotic embryos of peach against the culture filtrate produced by the bacterial pathogen *Xanthomonas compestris pv.pruni* and reported that regenerated plants were more resistant to the pathogen. Gentile [27] regenerated mal secco resistant lemon by selecting nucellar embryogenic cultures for resistance to a partially purified phytotoxin produced by the causal fungus *Phoma tracheifila*. Fusarium wilt disease resistant plants were regenerated from abaca embryogenic calli on medium fortified with *Fusarium oxysporum f.sp. cubense* culture filtrate [28]. *Phytophthora* resistant cultures of *Citrus jambhiri* were recovered by exposing cotyledon derived calli to culture filtrate obtained from the causal agent *Phytophthora parasitica* [29].

Objectives of the present study comprise i) Confirmation of tolerance/susceptibility of different *Hevea* clones towards CLFD through *in vitro* screening ii) Correlation of chitinase in imparting tolerance towards *Corynespora* pathogenicity iii) *In vitro* development of *Hevea* plants tolerant to CLFD using cassiicolin toxin.

## MATERIALS AND METHODS

### Plant Material

Eight prominent clones of *Hevea* identified as susceptible and tolerant to *Corynespora cassiicola,* from previous studies and field reports, were selected for studying the effect of cassiicolin under *in vitro* conditions. The clones selected were grouped as follows

Susceptible clones – RRII 105, RRII 203, PB 217 & PB 260

Tolerant clones –RRII 414, RRII 430, GT 1 & FX 516

Immature inflorescence was used as the initial explants [30] for callus induction from the above mentioned *Hevea* clones.

### Extraction of cassiicolin toxin from *Corynespora cassiicola*

Modified Czapeck liquid medium was used for cassiicolin production.100 ml of this liquid medium was placed in 500 ml flasks. Each flask was inoculated with three mycelia plugs (5 mm in diameter) from 7 day old culture of *C.cassiicola*. Liquid cultures were incubated without agitation at 25°C for 12 days and filtered under vaccum through a 0.22µm Millipore membrane. This culture filtrate is used as the crude toxin for the present study.

#### 1. *In vitro* confirmation of sensitivity of *Hevea* clones towards cassiicolin

**Laboratory screening using detached leaf technique**

Immature leaves (pale green stage) of both susceptible and tolerant clones were collected and cut into small pieces which were then subjected to vacuum infiltration with 2 ml of crude toxin for 15 minutes. These leaf samples were transferred to moistened filter paper in petri plates and kept at room temperature. Leaf pieces vacuum infiltrated with sterile water for 15 minutes served as control. Both control and toxin treated leaves were scanned at 12, 24 and 36 hrs interval for visualization of necrosis. For better visualization of necrotic areas the eye dropper tool was used whereby the colour difference between necrotic and non- necrotic tissues could be identified.

**_In vitro_ screening of callus against cassiicolin**

Immature inflorescence of eight clones of *Hevea* belonging to tolerant and susceptible groups under study were used as the initial explants for callus induction. Fresh, proliferated calli were exposed to cassiicolin by transferring to proliferation medium additionally supplemented with different volumes of filter sterilized crude toxin (0.5, 1.0, 2.0 and 3.0 ml/ 100 ml medium). Calli inoculated on toxin free proliferation medium were used as control. The

cultures were incubated under dark conditions at 25±2°C. After three weeks in culture, the effect of toxin was assessed through visual evaluation of various parameters such as percentage of necrosis, colour and morphological changes of the callus.

### 2. Estimation of chitinase activity in leaves

Pale green leaves of field grown plants belonging to the 8 clones under study were selected for this experiment. Leaf chitinase activity was measured in these clones using the following samples

a) Control samples – Fresh leaves collected directly from the field grown plants

b) Toxin treated samples – Application of toxin by leaf puncture technique, keeping the leaves on the plant itself for 15 hrs after which the leaves were harvested

c) Water treated control - Sterile water was applied instead of toxin and the leaves were kept on the plant itself for 15 hrs before harvesting

Comparison of chitinase activity in the control samples was carried out in order to see whether there is any difference in the enzyme activity between the susceptible and tolerant groups. Similarly the enzyme activity after toxin treatment was assayed in all the clones to determine the impact of exposure to toxin on individual clones. Also a comparison between the control and toxin treated samples of each clone was performed along with a water treated control.

#### Enzyme extraction and assay

The leaf tissue was homogenized in a pre-chilled mortar and pestle with 0.1 M sodium citrate buffer of pH 5.0 (1.0 ml/0.5 g fresh weight).The crude homogenate was then centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was collected and used for chitinase assay.

DNS method was employed for the measurement of chitinase activity. The reaction mixture containing 0.5 ml of 1% w/v chitin and 0.1 ml enzyme extract was incubated at 45 °C for one hour. Then 3 ml of 3, 5- dinitrosalicylic acid reagent was added for stopping the reaction followed by heating at 100 °C for 5 min. After cooling, determination of reducing sugar in the supernatant was accomplished by the modified method of Miller [31]. Absorbance was measured at 530 nm using UV spectrophotometer along with substrate and blanks.

### 3. *In vitro* selection for *Corynespora* tolerance using cassiicolin

RRII 105, the most popular and widely cultivated clone, which at the same time susceptible to CLFD, was selected for this study. Fresh, proliferating callus raised from immature inflorescence was challenged for 6 weeks on callus proliferation medium supplemented with different levels (0.1, 0.2, 0.5, 0.8 and 1% (v/v) of cassiicolin, designated as $T_1$, $T_2$, $T_3$, $T_4$ & $T_5$ respectively. After 6 weeks, the surviving calli emerged in the presence of different toxin levels were transferred to embryo induction medium and maintained in the dark at 25±2°C. Embryogenic calli and embryos induced in these media were subcultured for embryo germination and further plant regeneration.

## RESULTS AND DISCUSSION

### Detached leaf technique

It was observed that the intensity of damage after toxin treatment was well in accordance with the already established susceptibility/tolerance of various clones towards CLFD. In the case of susceptible clones (RRII 105, 203 & PB 217, 260) initial necrotic symptoms started appearing by 12 hrs of toxin treatment. Intensity of necrosis increased considerably with increase of exposure time, showing dark brown lesions followed by partial and complete death of tissue by 36hrs of treatment. Among the tolerant group, clones RRII 430, GT1 and FX 516 remained green without any necrosis even after 36

hours of toxin treatment, indicating tolerance of these clones towards the toxin. In the case of RRII 414, slight necrosis was noticed at 12hrs of toxin treatment. However there was only marginal increase in necrosis with increase in exposure time. The colour difference between necrotic and non necrotic tissue was made clear using eye dropper tool **(Fig. 1)**.

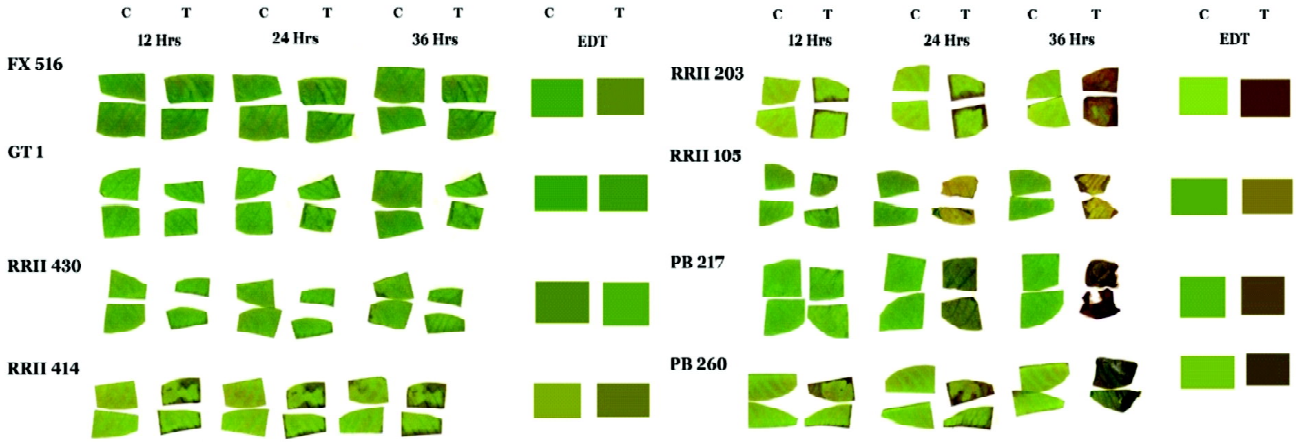


**Figure 1: Differential sensitivity of leaves of *Hevea* clones towards cassiicolin. C - water treated control, T - toxin treated, EDT - eye dropper tool at 36 Hrs**

## *In vitro* screening of callus against cassiicolin

It has been observed that in the toxin enriched medium, percent survival of calli from different clones under study showed marked difference. Moreover the response was well in accordance with the findings of the detached leaf technique. Overall growth and survival of intact callus was observed only in the tolerant group. Clones GT1 & FX 516 were found to be the most tolerant, being unaffected even at the highest level of toxin tried (3ml). Clones 414 and 430 showed tolerance upto 2ml of toxin as evidenced in **Fig. 2**. Regarding the susceptible clones,

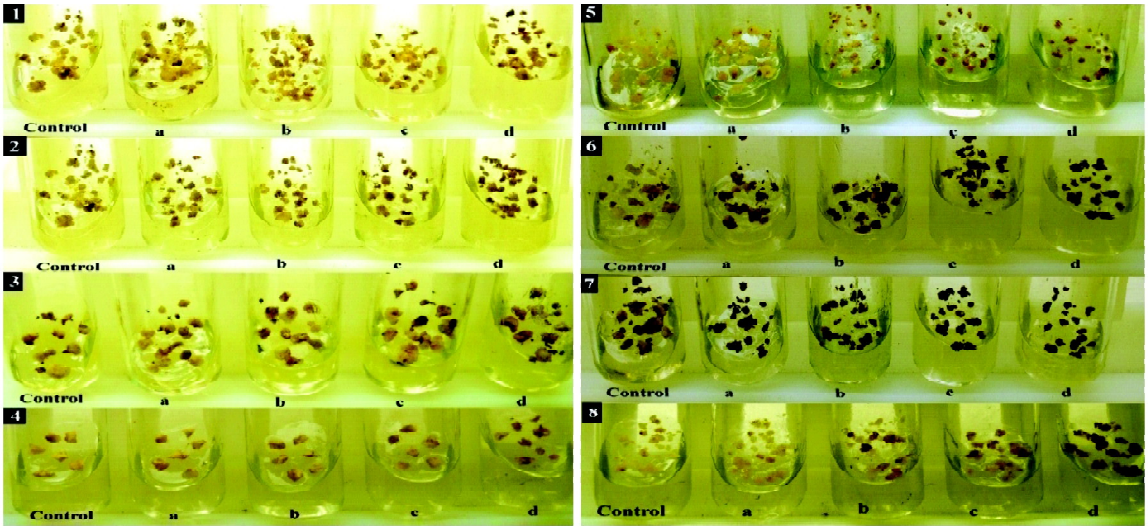


**Figure 2: Differential sensitivity of calli of *Hevea* clones (1-8) 1- RRII 414, 2 - RRII 430, 3 -FX 516, 4-GT 1, 5 - RRII 105, 6 - PB 217, 7 - PB 260, 8 –RRII 203 on exposure to cassiicolin at different volumes (a-d) a – 0.5 ml, b -1 ml, c -2 ml & d -3 ml of toxin/100 ml medium**

even the lowest level of toxin (0.5ml) was lethal to PB 217 and PB 260 while in the case of RRII 105 & 203, browning and necrosis of the calli was observed with 1ml toxin onwards.

## Estimation of chitinase activity

It has been observed that there was significant difference in the inherent chitinase activity of susceptible and tolerant clones, as evidenced in **Fig.3**. Chitinase activity was much low in all the four susceptible clones whereas all the clones belonging to the tolerant group exhibited significantly higher levels of chitinase. Among the tolerant clones, FX 516 showed the highest enzyme activity followed by GT1.

The same trend was observed among the different samples after toxin treatment, tolerant clones exhibiting higher enzyme activity than the susceptible ones (**Fig. 4**).

Comparison of chitinase activity within one clone before and after toxin treatment revealed that introduction of fungal toxin significantly increased the enzyme activity in the tolerant clones FX 516 and GT1.The increase was more predominant in FX 516 indicating this clone to be more tolerant

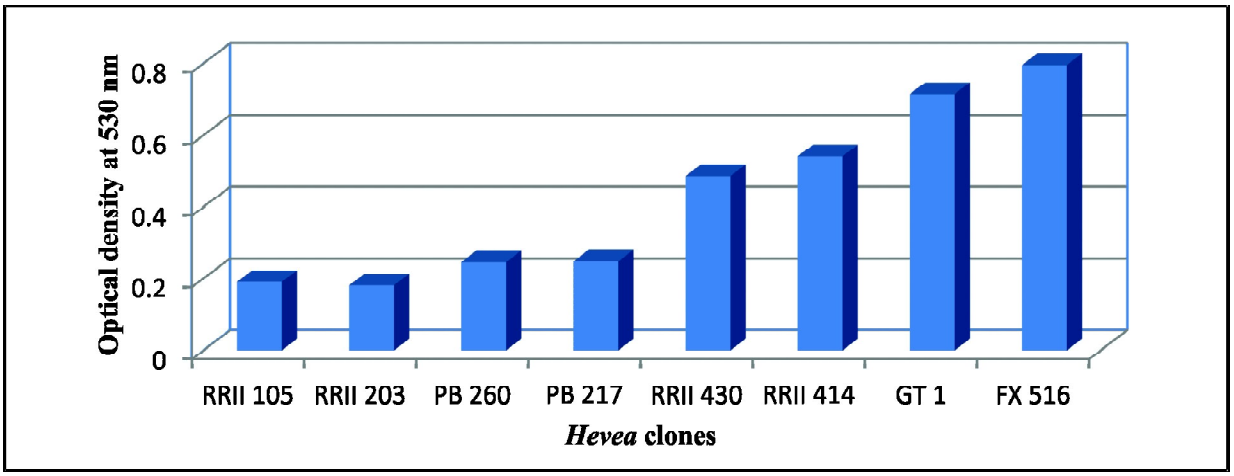


**Figure 3: Inherent chitinase activity in leaves of *Hevea* clones**

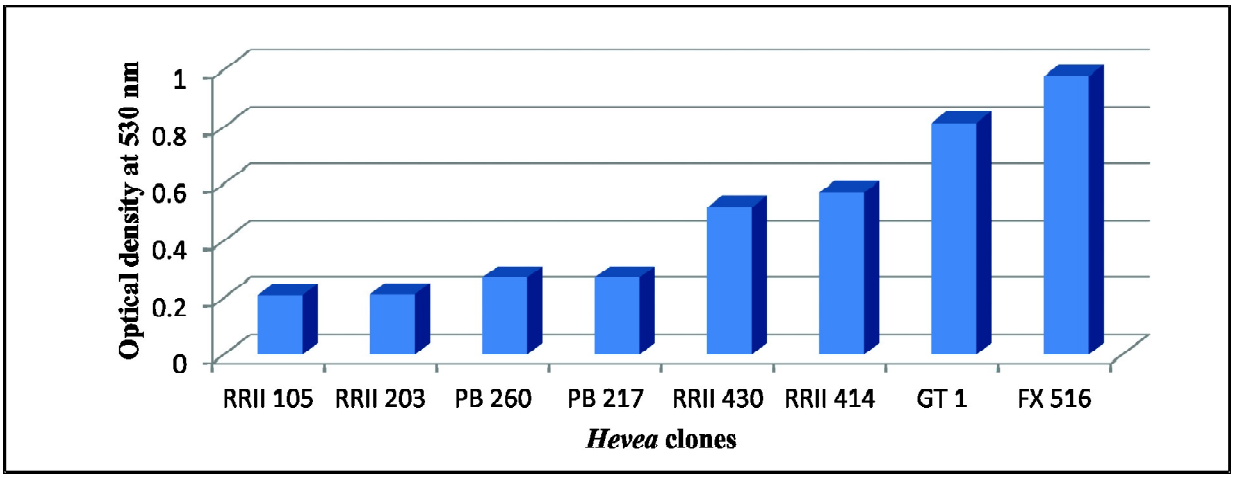


**Figure 4 : Chitinase activity in leaves of *Hevea* clonesafter toxin treatment**

towards the toxin. In the 400 series clones grouped under the tolerant category, no marked increase in the enzyme activity could be noticed. Similarly there was no significant increase in the chitinase activity in the susceptible clones upon toxin treatment **(Fig. 5)**.

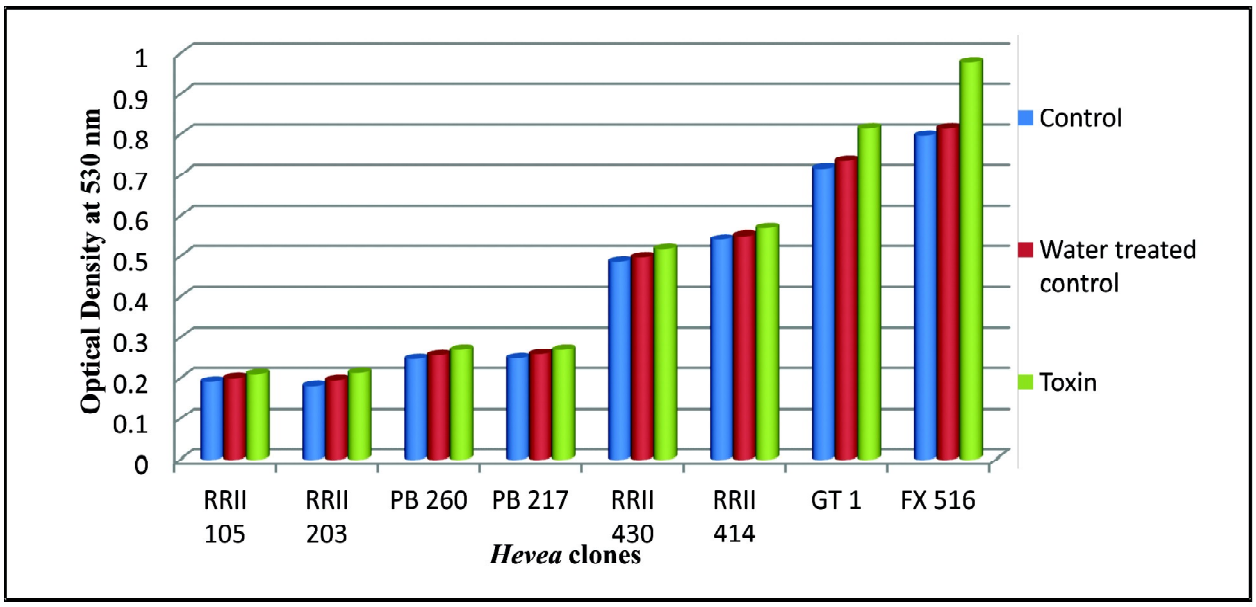


**Figure 5: Comparison of chitinase activity in leaves of different *Hevea* clones before and after toxin treatment**

## *In vitro* selection for *Corynespora* tolerance

It was observed that supplementation of toxin in the proliferation medium interfered with further development of the callus. Growth and proliferation rate of the callus decreased significantly as the concentration of crude toxin in the medium increased. As evidenced in the **Fig. 6**, callus growth and proliferation on $T_1$ medium was not at all affected, indicating that 0.1% toxin was not sufficient for inhibiting the callus growth. On the contrary, the growth of calli on $T_5$ medium containing the highest toxin level (1.0%) was completely inhibited as a result of which these calli turned brown and gradually dried up.However, a few calli survived to grow on intermediate toxin levels ($T_2$,$T_3$ & $T_4$), without becoming necrotic. After 6 weeks in the toxin medium, the surviving calli were transferred to toxin free proliferation medium and later subcultured on to embryo induction medium.Within 6-8 weeks some of these calli turned embryogenic and later gave rise to several embryos. Eventhough embryo induction could be obtained from the cultures developed from $T_2$, $T_3$ and $T_4$ media, there was considerable difference in further development of these embryos. Embryo germination and plant regeneration could be obtained from embryos emerged from $T_2$ and $T_3$ media whereas further growth of the embryos emerged from $T_4$ media got suspended. Those embryos remained stunted in the germination medium and later dried up.

Some of the cells within the callus tissue from $T_2$ and $T_3$ media might have acquired CLFD tolerance so as to survive in the presence of cassiicolin. Such cells might have proliferated, underwent differentiation and gave rise to embryogenic callus, embryos and finally plantlets.The reason behind a few cells acquiring disease tolerance may be attributed to somaclonal variation, indicating

that the selected cells might get altered and produce toxin suppressors or detoxifying enzymes which either suppress or detoxify the crude toxin. The comparatively higher toxin level in $T_4$ medium might have interfered with the normal development of the embryos raised from that medium. Plantlets obtained from $T_2$ and $T_3$ media were transferred to half strength regeneration medium, as a preparatory step for acclimatization.Induced tolerance of the regenerated plants, under field conditions, towards CLFD needs to be validated using biochemical and molecular techniques and also by exposure of the plants to *Corynespora.*

This method opens up an alternate avenue for imparting CLFD tolerance to otherwise susceptible clones. *In vitro* selection strategy facilitates the screening of many clones within a short period, in a relatively small space, by using crude or partially purified pathotoxin. Moreover, this technique is a promising non transgenic approach which offers an attractive alternative for *in vitro* production of disease tolerant clones.

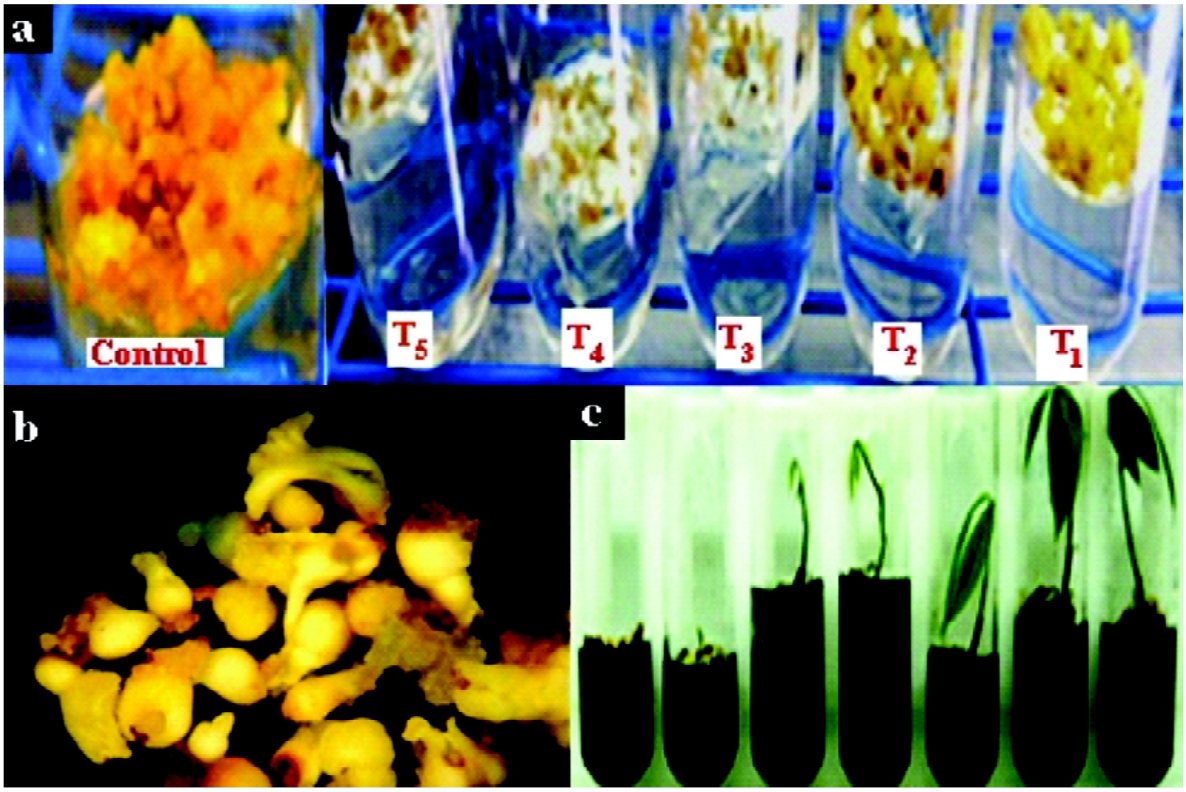


**Figure 6*: In vitro* selection of tolerant callus and plant regeneration**

a- Differential sensitivity of calli of *Hevea* clone RRII 105 on exposure to different levels of cassiicolin $T_5$-$T_1$ (1.0 -0.1 % v/v), b- Somatic embryos induced from the proliferated tolerant callus, c- Embryo germination and plant regeneration

## CONCLUSION

Inference from the *in vitro* screening experiments using detached leaves and callus cultures are clearly in conformity with the earlier observations and field reports regarding the sensitivity of different clones of *Hevea* towards CLFD. This indicates that both these methods can be employed in determining the degree of tolerance of a given clone towards CLFD. These two in *vitro* techniques, being quite simple and fast, can be employed in the early screening of new and pipeline clones towards CLFD.

Outcome of the chitinase activity measurement is also quite encouraging. Initial results obtained so far throws light on the positive role of chitinase in imparting tolerance towards CLFD. This information also can be made use of in the screening of newly developed clones towards this disease. Moreover the positive correlation between high chitinase activity and tolerance to CLFD opens up the scope of imparting CLFD tolerance to elite clones of *Hevea* by overexpression of chitinase gene through genetic manipulation techniques.

Toxin based *in vitro* selection study represents an economical and expeditious way of generating/ selecting plants with resistance to cassiicolin toxin from susceptible varieties, as compared to time consuming classical breeding methods. Moreover it is a promising, biosafe, non transgenic approach. In our present study a few plantlets have been regenerated from the callus exposed to cassiicolin, indicating the possibility of *in vitro* induced disease tolerance.

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