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Screening of Wheat Varieties for Fusarium Disease Using Cycloheximide (CHX) as Selection Agent in Culture Media

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Abstract: Fusarium Head Blight caused by *Fusarium* spp. results reduction in yield and grain quality by contamination of grain with mycotoxins mainly deoxynivalenol. Because of deoxynivalenol is very toxic and expensive, it is important strategy to substitute deoxynivalenol by another safe and cheap agent to screen wheat varieties on *in vitro* condition. Cycloheximide is a protein synthesis inhibitor and acts in same manner of inhibition which deoxynivalenol causes it. Therefore, it may be practical to use it for this purpose. In this research potentially application of cycloheximide was studied by using 6 wheat varieties with different resistance level to Fusarium disease in two separate experiments using deoxynivalenol and cycloheximide. These varieties have been screend for Fusarium head blight in field and greenhouse. Number of germinated seeds and Number of seeds with coleoptiles longer than 2 mm was recorded in media containing different concentrations of deoxynivalenol or cycloheximide. The results showed high positive correlation between two experiments and it is possible to use cycloheximide for screen wheat varieties for Fusaium in *in vitro* selection.

Key words: Cycloheximide (CHX), Deoxynivalenol (DON), FHB, *in vitro*

INTRODUCTION

Fusarium species, including *Fusarium graminearum* Schwabe {teleomorph: *Gibberella zeae* (Schw.) Petch} and *F. culmorum* (W.G. Smith) Sacc., cause root rot, seedling blight, foot rot and head blight (Fusarium head blight or FHB) diseases of small-grain cereals (Miedaner, 1997). These diseases are economically important because they cause a reduction in yield and grain quality. FHB disease caused by these pathogens also results in the contamination of grain with mycotoxins, mainly deoxynivalenol (DON) and zearalenone (Rocha *et al*., 2005).

DON is a mycotoxin of the trichotecenes group, one of the most spread mycotoxin in cereal, which can be synthesized in the field in winter cereal and in maize grain (Chelkowski 1989; Schaafsma *et al*., 1993). Trichotecenes are associated with serious mycotoxicosis in humans and animals. They have cytotoxic activity (protein synthesis inhibition, effects on DNA and RNA synthesis, mitochondrial function inhibition, effects on cellular membranes and on cellular correct division, and apoptosis) and immunosuppressor effect, which reduce the resistance to microbial infections (Rotter *et al*, 1996; Shifrin and Anderson, 1999; Minervini *et al*., 2004). At the cellular level, the main toxic effect of DON is inhibition of protein synthesis via binding to the ribosome. (Rotter *et al*., 1996). Deoxynivalenol (DON), known colloquially as "vomitoxin" (Canady *et al*., 2001), is most commonly detected, often at the ppm level (Abouzied *et al*., 1991; Lee *et al*., 1985; Rotter *et al*., 1996; Sugiura *et al*., 1990; Tanaka *et al*., 1990).

DON and other tricothecene mycotoxins contain some of the most potent protein synthesis inhibitors (Ueno 1987). Protein synthesis inhibitors have been shown to superinduce the expression of a number of genes (Edwards and mahadevan, 1992; Lusska *et al*., 1992; Mahadevan and Edwards, 1991). Superinduction can be defined as the capacity of protein synthesis inhibitors significantly to augment and prolong the usually transient mitogenic induction of a gene as a secondary consequence of translational arrest. There is lots of report to effect of DON on cytokine mRNA levels in mice and marked elevation of serum immunoglobin A (IgA) as well as other immunological perturbations (Pestka and Bondy, 1994; Efrat and Kaempfer, 1984; Warner and Libby 1989) following by enhanced production of interleukin 4 (IL-4), IL-5 and IL-6 (Warner et al., 1994). It was found that continuous exposure to DON or Cycloheximide (CHX) superinduces IL-2, -4, -5 and -6

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secretion at concentrations that partially inhibit protein synthesis in mice (Svetic *et al*., 1991; Azcona-oliveera *et al*., 1995). Azcona-oliveera *et al*. (1995) showed similar and even more marked effects for CHX and DON in gene expression and secretion of ILs in mice.

Therefore, it seems that CHX and DON inhibit protein synthesis in same biochemical way so it might be practical to apply CHX instead of DON for *in vitro* screening of wheat varieties against FHB. We have chosen some wheat varieties in different level of either susceptibility or resistance which come from two field and one greenhouse FHB evolution experiment. The main goal of this study was substitution of CHX instead of DON in *in vitro* screenings because CHX has less toxic effect for human rather than DON which is extreme vomitoxin agent.

MATERIAL AND METHOD

2-1) Plant materials:

Two different experiments were designed to evaluate DON and CHX as selective agent to investigate resistant level of wheat varieties for Fusarium in *in vitro* condition. Six varieties were selected for both experiments which had different response to Fusarium under field and greenhouse condition in previous studies. These variety contained sumyi-3, Falat, Pishtaz, Golestan, Moghan and Tajan. The only difference is Frontana variety which used in CHX experiment instead of Sumay-3 because both are resistant to Fusarium.

The media for both experiments was the same and prepared by using Na_2HPo_4, KH_2Po_4 and distilled water. Also agar was added to media and solvate by heating and shaking. The media was autoclaved and different amount of selection agent was added to separate part of media to obtain different concentration.

For each experiment, 150 sterilized seeds of each variety put in a separate dishes containing different concentration of selection agent and shacked overnight at room temperature and 100 rpm. 50 seeds of each variety were put on Petri dishes containing different concentration of selection agent in media. Each variety was repeated 3 times in each level of selection agent.

The Petri dishes was put in germinator at 5˚C. The number of germinated seeds ($NGTc_{80}$) and the number of seeds with coleoptiles longer than 2 mm ($NCTc_{80}$) was recorded daily until all Petri dishes reached to 80% of control in both traits. The method was the same for two experiments, the only differences was selection agent which added to media.

In first experiment, three levels of DON as well as control were used. The DON concentration levels consisted of 10, 15 and 20 ppm. But in second experiment, because of there is no information about best range of CHX concentration, the preliminary small experiment was designed to obtain the range of CHX concentrations. In this order, 10 fold concentrations from 0 to 10000 ppb of CHX were used on Falat as susceptible variety to FHB. Seed and media preparation was the same as described above and finally the germinated seeds were recorded. Based on the results the best range of CHX was determined as 0, 3, 6, 9 and 12 ppm.

2-2) Statistical Analysis:

The data was normalized by radically transformation and transformed data was used for analysis. The results was analysed in control level for two traits based on Complete Block Design (RCBD) and if there was significantly differences between varieties at control level, data was adjusted based on control to remove genetically differences between variety for both traits using formula 1.

Adjusted data=unadjusted data –(average of trait in control level for all variety- amount of trait for variety under analysis)

The data was analyzed based on RCBD for each selection agent level separately and all data was analyzed using Factorial design on bases RCBD to evaluation interaction effect between different varieties and different levels of selection agent in both experiments. Finally correlation coefficient was used to investigate the possibility of substitution of DON by CHX as a selection agent to select wheat variety for آإ disease in *in vitro* experiment.

RESULTS AND DISCUSSION

DON Experiment:

In DON experiment, ANOVA analysis in control level showed no significant differences between different varieties for both traitس and therefore there was no need to adjust data using formula 1. The results for ANOVA separately on each DON concentration based on RCBD showed significantly differences between

varieties for $NGTc_{80}$ and $NCTc_{80}$ in all level of DON concentration. As shown in table 1, in first level concentration of DON (10 ppm) Moghan and Falat had the highest and lowest NGTc80, respectively and other varieties were between them and didn't have significant differences with each other. Also Golestan had the highest $NCTc_{80}$ and Falat, Sumay-3 and Tajan had the lowest $NCTc_{80}$. In 15 ppm DON concentration, Sumay-3 and Moghan showed highest $NGTc_{80}$ and Falat had the lowest $NGTc_{80}$. In this level, Golestan showed the highest but Falat and Sumay-3 had the lowest $NCTc_{80}$. Finally in last concentration level of DON (20 ppm) Moghan showed the highest $NGTc_{80}$ but Tajan, Pishtaz and Falat had the lowest $NGTc_{80}$. In this level, Golestan had highest $NCTc_{80}$ and Sumay-3, Falat, Tajan and Pishtaz showed the lowest $NCTc_{80}$.

Table 1: Comparison of means for $NGTc_{80}$ and $NCTc_{80}$ in different level of DON

	10 ppm		15 ppm		20 ppm	
	$NGTc_{80}$	$NCTc_{80}$	$NGTc_{80}$	$NCTc_{80}$	$NGTc_{80}$	$NCTc_{80}$
Sumay-3	AB[1]	C	A	D	AB	C
Golestan	AB	A	AB	A	BC	A
Moghan	A	B	A	BC	A	B
Pishtaz	BC	B	BC	AB	C	C
Tajan	BC	C	AB	C	C	C
Falat	C	C	C	D	C	C

[1]. Means with at least one same letter don't have significant differences

Factorial analysis showed significant differences between varieties, DON level and interaction effect for both $NGTc_{80}$ and $NCTc_{80}$ trait in 1% probability level. Based on the result, Golestan had the highest and Falat had the lowest $NGTc_{80}$ and $NCTc_{80}$ over all DON levels. Also, considering all varieties, $NGTc_{80}$ and $NCTc_{80}$ was reduced significantly with increasing DON concentration in media culture.

Because the interaction between variety and toxin was significant in respect to $NGTc_{80}$ and $NCTc_{80}$, therefore NGTc80 and $NCTc_{80}$ changing pattern of varieties is not the same in different DON concentration, as shown in Fig 1 and Fig 2.

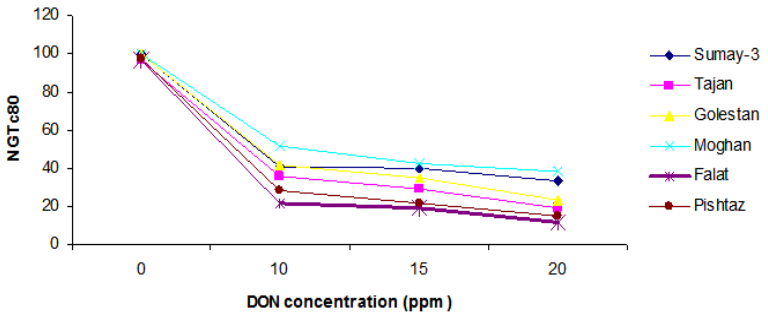

Fig. 1: $NGTc_{80}$ changes pattern of varieties under different concentrations of DON

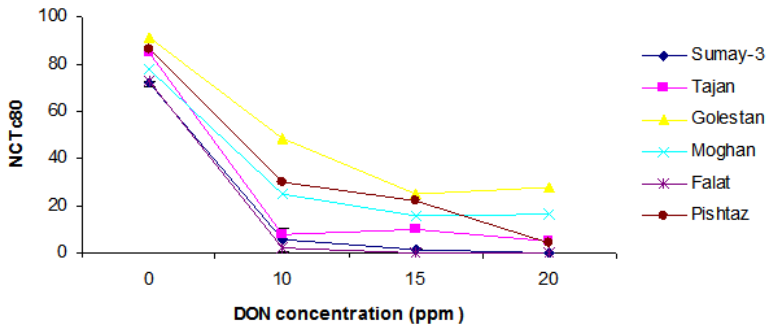

Fig. 2: $NCTc_{80}$ changes pattern of varieties under different concentrations of DON.

CHX Experiment:

Based on the result of preliminary test using different level of CHX on Falat, germination percentage was reduced between 1 to 10 ppm and therefore the best concentrations for experiment was determined between 1 to 10 ppm as 3, 6, 9 and 12 ppm as well as control.

The transformed data was analysed in control level for two traits based on Complete Block Design (RCBD) and varieties showed significant differences for two traits. Therefore the data was adjusted using formula 1 based on control to remove genetically differences between varieties for $NGTc_{80}$ and $NCTc_{80}$.

The ANOVA results for CHX experiment was shown in table 2. Based on the results all level of CHX could lead to significant differences between varieties for $NGTc_{80}$ and $NCTc_{80}$. In first level (3 ppm), Frontana and Golestan had highest NGTc80 without significantly differences together. The lowest $NGTc_{80}$ was for Tajan followed by Falat. Also Frontana and Golestan as well as Pishtaz had highest $NCTc_{80}$ and there was no significant difference between them and Tajan had the lowest $NCTc_{80}$ in this level.
In second (6 ppm), third (9 ppm) and forth (12 ppm) leval of CHX, Golestan and Frontana showed highest $NGTc_{80}$ and $NCTc_{80}$ and Tajan and Falat had lowest amount in both traits.

Table 2: Comparison of means for $NGTc_{80}$ and $NCTc_{80}$ in different level of CHX

	3 ppm		6 ppm		9 ppm		12 ppm	
	$NGTc_{80}$	$NCTc_{80}$	$NGTc_{80}$	$NCTc_{80}$	$NGTc_{80}$	$NCTc_{80}$	$NGTc_{80}$	$NCTc_{80}$
Frontana	A[1]	AB	A	AB	A	A	A	AB
Golestan	A	A	A	A	AB	A	A	A
Moghan	B	BC	B	BC	BC	AB	B	BC
Pishtaz	B	AB	B	CD	C	B	BC	BC
Tajan	D	D	C	E	D	B	D	C
Falat	C	C	C	DE	D	B	CD	C

[1]. Means with at least one same letter don't have significant differences

The ANOVA result on Factorial design showed significant differences between varieties and CHX level for both traits and interaction effect was not significant. This result was predictable from mean comparison of varieties in different levels of CHX as described above. In all CHX levels, the varieties follow the same pattern and in all levels Frontana and Golestan had highest amount and Tajan as well as Falat had lowest amount of both traits (Fig 3 and 4).

Based on the results and because of the varieties showed significant differences in all level of CHX, the lowest level (3 ppm) can propose as a good selection agent for Fusarium in wheat varieties.

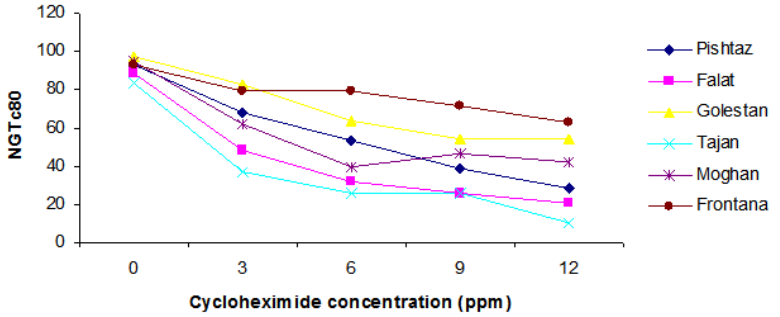

Fig. 3: $NGTc_{80}$ changes pattern of varieties under different concentrations of CHX.

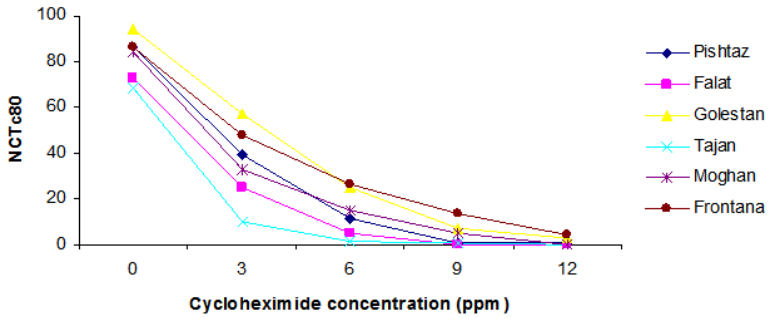

Fig. 4: NCTc80 changes pattern of varieties under different concentrations of CHX

In order to evaluate using CHX as a good selection agent instead of DON toxin in *in vitro* experiments to screen wheat varieties for Fusarium disease, the correlation between data from DON experiment and CHX experiment was calculated. The correlation coefficient between three first level of CHX and three level of DON was significant for $NGTc_{80}$ and $NCTc_{80}$ in 1% and 5% probability level, respectively.

Discussion:

Based on the result, CHX can propose as a good selector agent to substitute with DON toxin and 3 ppm of CHX is enough to evaluate resistance level of wheat varieties for Fusarium. Because of DON is very toxic and expensive and has very danger for environment, therefore it can be replace by another agent in *in vitro* Fusarium experiments. As shown in this study, CHX can be a good candidate to substitute by DON. CHX is an antibiotic and therefore it is not toxic at all and also it is very cheaper than DON. CHX can use in *in vitro* experiment without any side effecst for human and environment and it is available everywhere. The application of CHX as a protein synthesis inhibitor with same effect on some organisms like human and mice was reported earlier (Svetic *et al*., 1991; Azcona-oliveera *et al*., 1995) and in this study we introduce CHX as a selector agent in culture media to screen resistant level of different wheat varieties for Fusarium disease which would be cheaper and more safe than DON.

Abbreviation:

CHX: Cycloheximide
DON: deoxynivalenol
NGT_{c80}: Number of germinated seeds a
NCT_{c80}: Number of seeds with coleoptiles longer than 2 mm
FHB: Fusarium Head Blight

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