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Table of Content: Volume 8 Number 5, May 2014

ARTICLES

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Full Length Research Paper

The effect of salinity (NaCl) on germination and early seedling growth of *Lathyrus sativus* and *Pisum sativum* var. *abyssinicum*

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High salt level of a germination medium may induce a reduction, delay and even complete inhibition of germination due to osmotic effect and/or ion toxicity. The objective of this study was to investigate the effect of salinity due to NaCl on germination and early seedling growth of two crops, *Pisum sativum* var. *abyssinicum* and *Lathyrus sativus.* Seeds of these two crops were treated with NaCl induced saline germinating media prepared in Petri dishes. Fifty (50) surface sterilized seeds per Petri dish were sown in five salt treatments (0, 5, 7, 9 and 15 dSm^{-}). Each treatment was replicated four times. Germination percentage, shoot length and root length of both crops decreased with an increase in salinity level. Although both crops are low salt tolerant legumes, *P. sativum* var. *abyssinium* was found to be less tolerant than *L. sativus.* This study could be strengthened by further work under field conditions and also at mature vegetative and reproductive stages of the crops.

Key words: Germination time, water uptake percentage, salt tolerance, seedling biomass.

INTRODUCTION

Germination is the initial stage of a plant's life cycle and determines where and when a crop can be established. It is a complex metabolic process that oxidizes the lipids and carbohydrates within the seed and breaks down storage proteins in order to obtain energy and amino acids necessary for plant development (Almansouri et al., 2001). Seed germination and early seedling growth under saline conditions are considered as major factors limiting the establishment of crops (Kitajima and Fenner, 2000). Interaction between seedbed environment and seed quality is also important (Khajeh-Hosseini et al., 2003). Plant available water is restricted in soils containing excess sodium chloride, resulting in partial dehydration of cell cytoplasm. Such plasmolysis affects the metabolism of cells and functions of macromolecules and, ultimately, results in cessation of growth (Le Rudulier, 2005).

The effect of salinity on germination can be either by creating osmotic potential which prevents the uptake of

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water or by the toxic effects of ions on embryo viability (Houle et al., 2001). Salts absorb and retain water with such strength that it is not freely available in the soil, causing an increase of soil solution osmotic pressure. Salt stress may cause significant reductions in the rate and final germination percentage (FG %), which in turn may lead to an uneven stand establishment and a reduction in yields.

Rapid, uniform and a high germination percentage for legumes is a prerequisite for successful stand establishment and yield (Demir and Ermis, 2003). The specific ions likely to be most abundant and cause greatest problems are sodium (Na^+) and chloride (Cl^-).

The legume family is the third largest of flowering plants (Morris et al., 2003; Lewis et al., 2005). Economically, legumes represent the second most important family of crop plants after Poaceae (grass family), accounting for approximately 27% of the world's crop production (Graham and Vance, 2003). Legumes, in developing countries, are largely produced as subsistence crops by small hold farmers. Ethiopia is a good producer of food legumes after Egypt in Africa (Lewis et al, 2005). However, about 10,608 ha of Ethiopia's total land is affected by salinity in semi-arid and arid regions (Geressu, 2011), which may reduce yield. Moreover, irrigated lands in the semi-arid parts of the country are increasingly becoming saline and turning to a new scenario of hampering food production for the fast growing population.

Reclaiming salt affected land is always costly and time consuming (Turan et al., 2007). Therefore, in countries like Ethiopia where legumes are principally grown for human consumption; the focus should remain on the development of salt tolerant cultivars to attain food security of the rapidly growing population. For this purpose, screening of suitable crops and cultivars is necessary. Identification of salt tolerant crops and cultivars is important so that such unproductive land can be used. Although, to our knowledge, there is no previous data available on *L. sativus* and *P. sativum* var. *abyssinicum* response to salinity in Northern Ethiopia. The objective of this study was therefore to investigate the effect of salinity on germination and early seedling growth of *P. sativum* var. *abyssinicum* and *L. sativus*.

MATERIALS AND METHODS

This study was conducted from October 2012 - March 2013 in the Laboratory of Botanical Sciences at the Department of Biology, Bahir Dar University, Ethiopia. Sterilized, hand selected seeds of both *L. sativus* (from Adet, Amhara Region) and *P. sativum* var *abyssinicum* (from Maichew, Tigray Region) were used for the study. Before beginning the experiment, solutions were made by dissolving sodium chloride in distilled water at five different concentrations (0, 5, 7, 9 and 15 dSm^- EC) (Li, 2008) and left for 48 h in order to dissolve. The salt solutions were prepared every six days so that it is relatively fresh for the germinating seeds.

Following the solution preparation, Petri dishes were washed and disinfected with alcohol and air dried. Germination chambers were prepared from the sterilized glass Petri dishes and filter papers (Whatman No. 2). The filter papers were cut into two pieces of equal size with labeled seed compartments and concentrations of salt solutions; and made ready for use. Treatments were arranged in a completely randomized design (CRD) and each treatment combination was replicated four times.

Dry weight of the hand selected seeds were recorded before the seeds were surface sterilized with 5% sodium hypochlorite solution for 10 min and rinsed with sterile distilled water three times. Fifty seeds were sown in each Whatman filter paper bedded dishes (Kaya et al., 2005). The seeds were watered with the appropriate salt solutions and left for 24 h where after, the fresh weight was recorded to calculate the uptake of water by the seeds. This was done as follows (Gairola et al., 2011):

Water uptake percentage =

$$\frac{\text{Seed fresh weight} - \text{Seed dry weight}}{\text{Seed fresh weight}} \times 100$$

After the measurement of water uptake, the seeds were re-sown into the Petri dishes. They were then watered daily with the appropriate salt solutions. All the germination and early seedling growth parameters were evaluated using the method used by Li (2008) with some modifications. Counting germinated seeds started 24 h after sowing every day for 14 days. A seed was considered to be germinated when plumule and radical emerge from the seeds. In all treatments, a continuous assessment in seedling growth were carried out during the subsequent days for both study crops, until day 14.

Germination rate (GR), which is important for state of readiness for early seedling growth under laboratory condition was evaluated as follows:

Germination rate = $(X_n - (X_n\text{-}1))/Y_n$

where, X_n is the number of germinated seeds at the n^{th} day and y_n is the number of days from sowing until the n^{th} harvesting time.

Germination Percentage (GP %): (GP) =

$$\frac{\text{Number of germinated seeds}}{\text{Total number of seeds sown}} \times 100$$ (Kandil et al., 2012).

Mean germination time (MGT): MGT = $\frac{\sum dn}{\sum n}$ (Kandil et al., 2012)

Where n is the number of seeds which germinated on day 'd', and 'd' is the number of days counted from the beginning of germination.

Germination index (GI): was calculated as the product of number of days after sowing and number of germinated seeds divided by the total number of sown seeds.

$$GI = \frac{\sum diNi}{S}$$ (Li, 2008)

where 'di' is number of days after sowing of seeds under a particular treatment, 'Ni' is number of germinated seeds, 'S' is total number of sown seeds of the study legume crops.

Seedling height reduction (SHR): the delay in root length and shoot length expressed in percentage was calculated as:

$$\frac{\text{Plant height at control} - \text{plant height at salt treat condition}}{\text{Plant height at control}} \times 100$$

(Morris et al., 2003)

Relative NaCl injury rate was calculated as the difference between germination percentage in control and germination percentage in salt treated seeds dividing by the germination percentage in the untreated seeds. The emergence of radicle (root) and shoot (plumule) of *P. sativum* and *L. sativus* from each Petri dish were assessed every day after sowing. And then the salt tolerance rate was calculated by using the standard formula used by Kaymakanova (2009):

$$\text{Salt tolerance (ST)} = \frac{\text{Seedling dry weight of treated}}{\text{Seedling dry weight in control}} \times 100$$

Data analysis

All collected data for germination and early seedling growth were organized, analyzed and interpreted using SPSS 20 for Windows and Microsoft excel softwares. Variance were statistically computed by analysis of variance (ANOVA) for the complete random design using SPSS version 20, and comparisons of means were made using the Tukey HSD significant difference test (HSD) at $P<0.05$. Graphics and tables were used to show the distribution of the response of the seeds to the different treatments.

RESULTS AND DISCUSSION

Water uptake percentage

Entry of water into seeds is greatly influenced by the nature of the seed coat (pericarp). Seeds of *L. sativus* and *P. sativum* var. *abyssinicum* respond differently though they are treated with the same salt concentrations. The results in Table 1 indicate that water uptake percentage increased in seeds of *L. sativus* and *P. sativum* treated with higher salt concentrations as compared to those treated with distilled water. In both seeds, the ability to absorb water is influenced by the concentration of salts and index of reduction increases with increasing salinity level from 9 to 15 dSm^{-}.

Seeds of *P. sativum* showed a high percentage of water uptake increment in all NaCl solution treatments except at the 7 dSm^{-} as compared to that of *L. sativus* seeds (Table 1). The overall water uptake percentage was not, however, statistically significant ($p < 0.05$) between the crop types and among the different salt concentrations except at 5 dSm^{-} for *L. sativus* (Table 1). This is as a result of large sample size.

Germination percentage

The interaction between the crops and salt concentrations was significant, meaning that the crops respond differently at specific salt concentration. This is shown by the significant differences ($P < 0.001$) in germination percentage of the two crop types given in all treatments (Table 1). Increased salt concentration caused decrease in germination percentage, but the extent of reduction under high concentration stress was much greater than that under low salt stress (Table 1).

Under the highest salt concentration (15 dSm^{-}), no seed germinated for either crop. Germination percentage of *L. sativus* was significantly ($p < 0.05$) higher than *P. sativum* var. *abyssinicum* at all salt concentrations (Table 1). This suggests that seeds of *L. sativus* could germinate well at a relatively higher concentration of NaCl than *P. sativum* var. *abyssinicum* seeds.

Germination rate

Differences among NaCl treatment were statistically significant ($P<0.0001$) for germination rate of the study seeds. The germination rate decreased as salt concentration increased to a 9 dSm^{-} and delayed for the highest salt dosage (Table 1). Since the higher salt concentration limited the water absorption, it slows down the germination rate. Higher germination rate was recorded for *L. sativus* seeds at 0, 5, and 7 dSm^{-} salt concentrations as compared to *P. sativum* var. *abyssinicum.* No seeds germinated at the highest salt concentration (15 dSm^{-}) for both species. Since higher salinity limited water absorption, it prevents nutrient assimilation, as a result, germination rate declined with increasing salinity. The results of this study were similar to the findings of Akhtar and Hussain (2008) and Kaydan and Yagmur (2008) who worked on different plant species. For example, Akthar and Hussain (2008) reported that germination of three grasses (*Bothriochlo apertusa, Dichanthium annulatum* and *Panicum antidotale*) significantly declined even at 5 dSm^{-} level of treatment.

Mean germination time

Salinity had a considerable increasing effect on germination time of *P. sativum* var. *abyssinicum* and *L. sativus* up to a certain level (Table 1). The increasing effect was more for *L. sativus* as compared to *P. sativum* var. *abyssinicum.* The highest average mean germination time was obtained from seeds treated with the 5 dSm^{-} salt concentration. In this study, mean germination time for *P. sativum* var. *abyssinicum* seeds at 5 dSm^{-} was higher than the control. This difference is significant at $p< 0.05$. There was also a significant difference ($P < 0.001$) in mean germination time of the two crops at 0, 5 and 7 dSm- salt concentrations. It means that the higher the salt concentration the longer the germination time until seeds develops tolerance and starts to germinate by overcoming the germination time delay (Patto, 2009).

L. sativus seeds took much time for germination than *P. sativum* var. *abyssinicum* seeds though both of them increased in mean germination time with increased salt solution treatment up to 5 dSm^{-}. That means seeds of *L. sativus* can tolerate salinity effect up to 9 dSm^{-} than *P. sativum* var. *abyssinicum* seeds. This shows that *L. sativus*

Table 1. Means comparison for effects of salinity (NaCl) on water uptake, percent germination, germination index, germination rate and mean germination time of *L. sativus* and *P. sativum* var. *abyssinicum.*

NaCl (dSm^-)	Water uptake (%)		Germination (%)		Germination index		Germination rate		Mean germination time (day)	
	L. sativus	***P. sativum***	***L. sativus***	***P. sativum***	***L. sativus***	***P. sativum***	***L. sativus***	***P. sativum***	***L. sativus***	***P. sativum***
0	84.83^{ab}	55.34^{ab}	99.9^{a}	84.40^{bc}	4.31^{a}	3.92^{ab}	13.22^{a}	12.86^{a}	2.91^{ab}	1.98^{c}
5	91.95^{ab}	73.89^{ab}	81.90^{b}	38.55^{d}	5.45^{a}	2.73^{bc}	10.34^{b}	5.44^{c}	3.63^{a}	2.26^{bc}
7	87.72^{ab}	71.90^{ab}	65.95^{c}	18.85^{ef}	5.24^{a}	1.11^{d}	8.66^{b}	2.82^{de}	3.50^{a}	0.74^{d}
9	86.45^{ab}	81.30^{ab}	22.10^{e}	5.90^{gf}	1.37^{cd}	0.58^{d}	3.53^{cd}	0.92^{ef}	0.91^{d}	0.38^{d}
15	89.11^{ab}	84.70^{ab}	0.00^{g}	0.00^{g}	0.00^{d}	0.00^{d}	0.00^{f}	0.00^{f}	0.00^{d}	0.00^{d}

Superscripts within the means of each column (a-f) with different letters indicate significant difference among means ($p<0.05$, using Tukey HSD test).

seeds are capable of germinating if the adverse effect of salinity is not extreme during their dormancy period. Delayed germination causes increased irrigation cost and irregular and weak seedling growth in the establishment of legume crops. Relevant results were reported by Gunjaca and Sarcevic (2000) and Almansouri et al. (2001). They reported that increasing osmotic potential decrease water uptake and slow down germination time.

Germination index

The germination index of both crops decreased significantly with increasing NaCl concentration (Table 1). The reduction gets stronger particularly at the higher level of salt concentration when compared with the control. Thus, germination index and NaCl concentration were negatively correlated. *L. sativus* was less affected by salt treatments as it has long, hard and penetrating root system than *P. sativum* var. *abyssinicum*. On the other hand, *P. sativum* var. *abyssinicum* seeds were more sensitive to the same salinity levels. In this crop, increase in salt concentration caused higher decreases in germination index values as compared to *L. sativus* (Table 1).

Generally, increase in salt concentration decreased the germination index of the study crops and this is in line with the findings of Khayatnez and Gholamin (2011) in *Zea mays* that showed decreased germination index as salt concentration increased. Previous reports from other workers also corroborate these results, for instance, Khan et al. (2009) reported on hot pepper. In this study, germination index of *P. sativum* var. *abyssinicum* decreased significantly with increasing NaCl concentration (Table 1). The reduction gets stronger particularly at 9 dSm^- NaCl concentration as compared to the control. Total decline in germination was observed at the highest salinity level (15 dSm^- NaCl). An increased germination index is indicative of decreased phytotoxicity and thus of a more mature germinated seeds (Khayatnezhad and Gholamin, 2011). However, results obtained using the germination index should be interpreted with care because they are affected by the type of seed used and the source of salinity. Application of damaged seeds and non-stabilized salts ($CaCl_2$) to germinating seeds may lead to immobilization of plant nutrients and cause phytotoxicity (Khan et al., 2009).

Effects of salinity on early seedling growth

Generally, germination and early seedling growth declined with increasing salinity levels, although reduction in root length was higher than reduction in shoot length. With higher salt concentration seedling growth rate declined in both crops. Root elongation was sensitive than shoot under the stresses on both seeds. It was affected at the lowest salt stresses (5 and 7 dSm^-) and was completely inhibited at the highest salt (15 dSm^-) concentrations (Table 2). As the osmotic pressure decrease at the germination environment, water cannot carry most of the water soluble nutrients to the root in order to increase osmotic pressure of roots to get enough water. Similar results were also found in older seedlings of chick pea by Li (2008). Thus, salinity had a deleterious effect and even total decline in seedling growth rate as the level of salinity increased beyond the tolerance range which could have resulted from a lower absorption of salt component by seed, and

Table 2. Mean comparisons for effects of salinity (NaCl) on root length, root height reduction, root fresh weight, root dry weight, shoot length and shoot height reduction of *L. sativus* and *P. sativum* var. *abyssinicum*.

NaCl (dS/m$^-$)	Root length (cm)		Root height reduction (%)		Root fresh weight (g)	
	L. sativus	*P. sativum*	*L. sativus*	*P. sativum*	*L. sativus*	*P. sativum*
0	10.26^{a}	8.03^{b}	0.00^{d}	0.00^{c}	7.4^{a}	6.7^{a}
5	4.13^{c}	2.01^{c}	57.37^{c}	74.34^{bc}	6.5^{a}	4.55^{b}
7	1.47^{cd}	0.55^{d}	57.37^{c}	92.84^{ab}	3.7^{bc}	4.55^{b}
9	1.47^{cd}	0.55^{d}	93.32^{ab}	92.84^{ab}	3.7^{bc}	1.83^{cd}
15	1.47^{cd}	0.00^{d}	93.32^{ab}	100^{a}	3.69^{bc}	1.83^{cd}

NaCl (dS/m$^-$)	Root dry weight (g.)		Shoot length (cm)		Shoot height reduction (%)	
	L. sativus	*P .sativum*	*L. sativus*	*P. sativum*	*L. sativus*	*P. sativum*
0	2.85^{ab}	3.48^{a}	10.12^{a}	10.29^{b}	0.00^{e}	0.00^{a}
5	2.51^{abc}	2.28^{bc}	8.93^{b}	7.79^{b}	12.70^{de}	24.06^{cde}
7	2.51^{abc}	1.82^{c}	5.31^{c}	6.15^{cd}	45.58^{bcd}	40.72^{bcd}
9	1.68^{cd}	0.98^{de}	3.80^{cd}	4.51^{de}	71.06^{ab}	54.58^{bc}
15	0.00^{e}	0.00^{e}	0.00^{e}	.0.00^{d}	79.82^{ab}	100^{e}

Superscripts (a-e) with different letters indicate significant difference among means within the columns ($p<0.05$, using Tukey HSD).

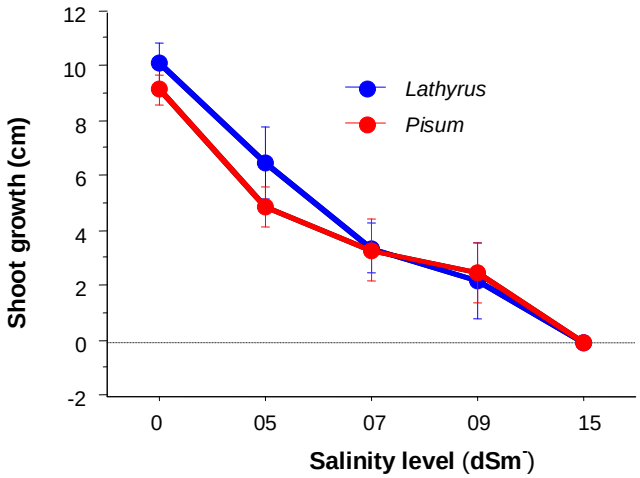

Figure 1. Seedling length of *L. sativus* and *P. sativum* var. *abyssinicum* grown under saline conditions at different stress levels. Error bars ± standard deviation.

germination process is also less responsive to high tissue sodium concentrations than seedlings growth (Figure 1).

The results of the present study showed that *P. sativum* var. *abyssinicum* and *L. sativus* roots during early seedling growth were more markedly inhibited than shoots under same salt stress conditions (Table 2). When compared with shoot, root elongation is more sensitive to the stress and is injured more severely (Table 2) because they are the first organs to face the stress. The injurious effect caused by salt stress was much greater on *P. sativum* var. *abyssinicum* than *L. sativus* (Table 2).

Sowing *P. sativum* var. *abyssinicum* under 7 dSm$^-$ salt stress had recorded lower root elongation than *L. sativus* seeds with significant difference between the two legumes ($p<0.01$). This species was highly affected as salinity stress level increases. However, both study crops at higher salinity levels, that is 9 dSm$^-$ produced the shortest roots with significant differences between them. In addition, results showed that maximum root length was obtained from sown *L. sativus* in the control treatment (Table 2).

Means comparison indicated that a significant reduction of leaf number was observed between seed types when salinity stress was increased from 0 to 9 dSm$^-$ while there

Table 3. Means comparison for effects of salinity (NaCl) on shoot dry weight, leaf number, relative NaCl injury rate, shoot fresh weight, seedling biomass and salt tolerance of *L. sativus* and *P. sativum* var. *abyssinicum*.

NaCl (dSm$^-$)	Shoot dry weight		Leaf number		Relative injury rate	
	L. sativus	*P. sativum*	*L. sativus*	*P. sativum*	*L. sativus*	*P. sativum*
0	3.42^{ab}	2.95^{bc}	1.93^{a}	1.68^{ab}	00^{f}	8^{ef}
5	4.12^{a}	3.52^{ab}	1.48^{b}	0.79^{cd}	18de	56^{c}
7	2.24^{cd}	1.73^{de}	0.99^{c}	0.79^{cd}	34^{d}	78^{b}
9	2.44^{cd}	1.40^{e}	0.37^{de}	0.33^{e}	78^{b}	93^{ab}
15	0.00^{f}	0.00^{f}	0.00^{e}	0.00^{e}	100^{a}	100^{a}

NaCl (dS/m$^-$)	Shoot fresh weight		Seedling biomass		Salt tolerance	
	L. sativus	*P. sativum*	*L. sativus*	*P. sativum*	*L. sativus*	*P. sativum*
0	7.40^{a}	5.03^{a}	1.00^{a}	0.79^{ab}	100^{a}	100^{a}
5	8.8^{a}	7.40^{b}	0.46^{bc}	0.28^{cd}	84.19^{a}	83.86^{a}
7	4.83^{b}	3.36^{bc}	0.27^{cd}	0.10^{d}	55.10^{b}	49.01^{b}
9	5.06^{b}	2.88^{c}	0.27^{cd}	0.21^{cd}	59.95^{b}	39.18^{b}
15	0.00^{f}	0.00^{f}	0.00^{d}	-0.00^{d}	-0.00^{c}	-0.00^{c}

Superscripts (a-f) with different letters indicated significant difference among means within columns ($p<0.05$, using Tukey HSD).

was no significant difference between salinity levels of 0, 5 and 7 dSm$^-$. Increasing salinity to 9 dSm$^-$ NaCl decreased shoot fresh weight, shoot dry weight, leaf number, seedling biomass, salt tolerance and increased relative sodium chloride injury of the two crops (Table 3). Shoot fresh weight was significantly influenced ($P<0.05$) by salinity levels. The highest shoot fresh weight was obtained from 5 dSm$^-$ salinity level while the lowest weight was at 15 dSm$^-$. Plant growth was affected positively up to 5 dSm$^-$ salt levels. At this level shoot fresh weight was higher than the control. However, shoot fresh weight significantly decreased as salinity level increased above 5 dSm$^-$ (Table 3). This result was supported by the findings of Kaya et al. (2005) who showed that shoot fresh weight increased in low NaCl levels for some plants.

Salinity stress significantly ($P<0.05$) affected shoot dry weight as the salt concentration dosage increased. Shoot dry weight significantly decreased at salt levels over 7 dSm$^-$. The highest shoot dry weights obtained (4.12 and 3.52 g for *L. sativus* and *P. sativum* var. *abyssinicum,* respectively) were from 5 dSm$^-$ salinity level, which were statistically different from the control. The present findings are in agreement with the results of other researchers on Alfalfa and Ryegrass (Mohammadi et al., 2008; Pessarakli and Kopec, 2009).

Leaf number and seedling biomass

Means comparison indicated that a significant reduction of leaf number and seedling biomass was observed between seed types when salinity stress was increased from 0 to 9 dSm$^-$. At the higher salt concentration (9 dSm) the leaf number was extremely affected and made seedlings less thrived for the stress. Leaves were totally delayed at the highest concentration (15 dSm$^-$) for both crops (Table 3). Seedling biomass of the crops also reduced as salt concentration increased (Table 3). Rahman et al. (2008) reported such a reduction and the reasons were believed to be due to slow or less mobilization of reserve foods, suspending the cell division, enlarging and injuring hypocotyls with increase in salt concentration in Gina cultivar. When the two species were compared, seedling biomass was higher for *L. sativus* than *P. sativum* var. *abyssinicum*.

Relative sodium chloride (NaCl) injury rate

The injury rate at 15 dSm$^-$ was 100% for both species. Effects were significant at all salt concentrations except at 5 and 7 dSm$^-$ for *L. sativus* and at 7 and 9 dSm$^-$ for *P. sativum* var. *abyssinicum* (Table 3). Relative NaCl injury rate was found to be higher for *P. sativum* var. *abyssinicum* as compared to *L. sativus* seeds at the same salt treatments. There was a direct relationship between the salinity levels and injury rate on both crops. Injury rate increased with increasing concentration of salinity level. This was similar to earlier studies made by Hadush and Gebreslassie (2012) on *L. sativus* landraces.

Salt tolerance

The present study indicates that salt tolerance in early

seedling stage was not correlated with seed germination. There were germinated seeds at the highest concentration (15 dSm^{-}) but died upto three days after germination (Table 3). This shows germination stage is more saline tolerant, which could result from a lower absorption of salt components by the seed. Germination process is also less responsive to high tissue sodium concentrations than seedlings growth. The result agrees with that of Mahdavi and SANAVY (2007) for *Schinopsis quebracho* where seeds at germination stage were relatively tolerant to salinity that at a later seedling stage. There was no tolerance at highest salinity (15 dSm^{-}) in both species. The difference in response to salinity shown by the two species at early stage of development may be related to their morphological and physiological variation. Results by Munns and James (2000) showed that crop plants could be salt tolerant at germination but turned salt sensitive during vegetative development.

Conclusion

Salt (NaCl) stress through enhancement of osmotic pressure leads to the reduction of germination percentage, germination rate, germination index and increase in mean germination time of *P. sativum* var. *abyssinicum* and *L. sativus* seeds. Salinity disturbed the metabolic and physiological processes starting from the imbibitions stage. *L. sativus* seeds were different from *P. sativum* var. *abyssinicum* for all the studied germination and seedling growth traits. Germination percentage decreased significantly with increasing salt concentration (≥ 7 dSm^{-}). Dry and fresh weight of seedlings decreased as seedling length declined with increasing salinity levels because root number, shoot number, root length and shoot length decreased significantly. The injurious effect caused by salinity stress was much greater on *P. sativum* var. *abyssinicum* than *L. sativus*. Roots of the early seedlings of *P. sativum* var. *abyssinicum* and *L. sativus* were more markedly inhibited than shoots under salt stress conditions. Salt tolerance in early seedling stage was not correlated with later developments. The results indicated the availability of salinity tolerant crops (to medium salinity) that could be useful in future planning for saline environments.

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Effect of seed-borne fungi on germination and seedling vigour of watermelon (*Citrullus lanatus* thumb)

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The effect of *Mucor racemosus* and *Rhizopus nigricans* inoculation (g and 0.1 g L^{-1} distilled water) and a seed dressing fungicide Seedplus® (1.25 g 0.5 kg^{-1} seeds) on germination and seedling vigour of watermelon (cv. Chaliston gray) was investigated. The experiment involved a complete randomized design (CRD) with three replicates. It was confirmed that the combined inocula with higher density caused significantly poor germination and low seedling vigour (P ≤ 0.05) than those seeds inoculated with lower inocula density. Higher germination percentage and seedling vigour were observed with single fungus inoculation than with the two fungi inoculation. Dressing of the fungi-infected seeds with Seedplus® 30 WS significantly improved germination percentage and seedling vigour of watermelon (P ≤ 0.05) but not as high as that of the control.

Key words: *Mucor racemosus*, *Rhizopus nigricans,* Seedplus.

INTRODUCTION

In Nigeria, the largest production of watermelon (*Citrullus lanatus* Thumb Family Cucurbitaceae) comes from the northern part of the country, where the suitable agro-ecology is found (IITA, 2007). Watermelon is cultivated extensively for its pleasant-tasting nature. Its fruits are mostly threatened by some pathogenic fungi which are seed-borne and also soil-borne such as *Mucor* spp. and *Rhizopus* spp. (Pamela and Tom, 2006). The effects of such fungi on the seedlings include poor germination, low seedling vigour and even complete failure of seedlings. These usually result in low yield and low income arising from poor yield quantity and quality.

Although much work has been done on investigating and discovery of fungal diseases that affect the fruits, there are no substantial report on the effect of pathogenic fungi on the seeds and seedling of watermelon. *Rhizopus stolonifer*, a type of black mould, has a wide host range and can affect over 300 plant species including fruits, vegetables and ornamentals (Farr et al., 2007). *Mucor* spp. which is a soil-borne pathogen may infect the fruit and stem of several plants such as pears and apple (Michailides, and Spotts, 1990). This study assessed the effect of *Mucor racemosus* and *Rhizopus nigricans* individually and in combination, and confirmed the efficacy of Seedplus® fungicide on germination and seedling of infected watermelon seeds.

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Table 1. Effects of inoculum density and types of pathogenic mould on germination percentage of watermelon.

Treatment	Germination percentage			
	5DAS (%)	6DAS (%)	7DAS (%)	8DAS (%)
M. racemosus + *R. nigricans* (g L^{-1})	46.67^{b}*	46.67^{c}	46.67^{c}	46. 67^{c}
M. racemosus + *R. nigricans* (0.1gL^{-1})	60.00^{ab}	60.00^{bc}	60.00^{bc}	60. 00^{bc}
M. racemosus (g L^{-1})	86.67^{a}	93.33^{a}	93.33^{a}	93.33^{a}
R. nigricans (g L^{-1})	73.33^{ab}	73.33^{abc}	73.33^{abc}	73.33^{ab}
M. racemosus (0.1 g L^{-1})	86.67^{a}	93.33^{a}	93.33^{a}	93.33^{a}
R. nigricans (0.1 g L^{-1})	86.67^{a}	93.33^{a}	93.33^{a}	93.33^{a}
M. racemosus + *R. nigricans* (g L^{-1}) + Seedplus®	78.00^{ab}	80.00^{ab}	80.00^{ab}	80.00^{ab}
Control	88.33^{a}	93.33^{a}	93.33^{a}	93.33^{a}

*Within each column, means followed by the same letter(s) are not significantly different (P ≤ 0.05) according to Duncan Test. **DAS, Days after sowing.

MATERIALS AND METHODS

Collection of seed sample

Watermelon seeds (cv Chaliston gray commonly grown in Nigeria) were obtained from Gwadabe market, Minna, Nigeria (Lat. 4° 30^{1} N of the Equator and Long. 10° 30^{1} E) where most farmers procure their planting seeds.

Inoculum production and identification

Confirmed strains of *R. nigricans* and *M. racemosus* by CABI Biosciences Identification Services (IMI 392668 & 392668) were maintained on cowpea seeds kept in Biochemistry Laboratory. The infected seeds samples were aseptically placed in 90 mm diameter Petri dishes containing 15 ml each of an autoclaved Potato Dextrose Agar, (PDA, Oxoid) added with 0.05 g l^{-1} chloramphenicol. It was incubated in lamina hood at 28°C and examined from 2-3 days in order to obtain the pure culture of the inoculum. On the third day after incubation, hyphal fragments inoculum was prepared by flooding the surface of the agar slant with sterile distilled water and gently scraping the surface of the sporing surface with a loop. Hyphal structures which are germ tubes were at least five times as long as the diameter of the spores. The resulting suspension with spores was then filtered off through sterile gauze. To reconfirm the identity of the fungi, mycelia speck from each colony were aseptically placed on a slide, stained with lactophenol blue, covered with slips and viewed under microscope (40x). The identification was accomplished using fungi catalogue in the Microbiology Department in F.U.T, Minna.

Inoculum quantification and inoculation

The hyphal strands of each fungus were diluted by adding sterile distilled water 1 g and 0.1g l^{-1} water to obtain the working suspensions. One hundred seeds (5 g) were pre-inoculated with 2 ml of each of the hyphal suspension concentrations by soaking for 20 min, that is, 0.4 L kg^{-1} seeds. The treatments included seeds pre-inoculated with high and low density of *M. racemosus* and *R. nigricans* (g 1 L^{-1}) and 0.1g L^{-1} distilled water as follows:

M. racemosus (g L^{-1}) + *R. nigricans* g L^{-1}; *M. racemosus* (0.1 g L^{-1}) + *R. nigricans* (0.1 g L^{-1}) *M. racemosus* (g L^{-1}), *R. nigricans* (g L^{-1}), *M. racemosus* (0.1 g L^{-1}), *R. nigricans* (0.1 g L^{-1}) and *M. racemosus* g L^{-1}+ *R. nigricans* (g L^{-1}) plus (10% imidacloprid + 10% metalaxyl + 10% carbendazim 2.5 g kg^{-1} seeds (Seedplus® 30 WS, Jiangsu Flag Industry Co., Ltd, Nianjing, China) and the control with uninoculated seeds.

A completely randomized experimental design with three replicates was involved. Ten seeds each were placed in 24 Petri dishes containing three layers of blotters moistened with 10 ml distilled water, set to germinate at 28 ± 2°C in the incubator and observed for eleven days for germination, fungal infection and seedling vigour.

Germination percentage, length of root and length of plumule were recorded daily as from the fifth to eleventh day. Germination percentage, length of radicle and length of plumule were recorded daily as from 5 to 11 days after sowing (DAS). Vigour index was calculated at 8 DAS according to Randahawa et al. (1985) and modified as follows:

$$VI = (PL + RL) \times GP$$

Where VI = Vigour index; PL = plumule length (cm); RL = radicle length (cm); GP = germination percentage (%).

Data collected were subjected to analysis of variance (ANOVA) and means were separated with Duncan multiple range test (DMRT) and *P* values <0.05 were considered statistically significant.

RESULTS

At five days after sowing (DAS), the germination percentage of watermelon seeds inoculated with high inoculum density (*M. racemosus* + *R. nigricans* (g L^{-1}) had the lowest percentage germination (46.67%) (Table 1).

The highest germination percentage was observed in the control (88.33%) and this was significantly different (P ≤ 0.05) from the seeds applied with *M. racemosus* + *R. nigricans* (gL^{-1}). At 6, 7 and 8 DAS, germination percentage of seeds applied with *M. racemosus* + *R. nigricans* (g L^{-1} or 0.1gL^{-1}) was significantly lower (P≤ 0.05) than that of other treatments (Table 2).

At 5 DAS, length of plumule (2.45 cm) from the seeds applied with *M. racemosus* + *R. nigricans* (g L^{-1}) was significantly lower (P≤ 0.05) than those applied with *M. racemosus* + *R. nigricans* (0.1g L^{-1}). The plumule length

Table 2. Effect of inoculum density and types of pathogenic mould on plumule length of watermelon seeds.

Treatment	Plumule length (cm)			
	5 DAS**	6DAS	7DAS	8DAS
M. racemosus + *R. nigricans* (g L^{-1})	3.45^{b*}	3.55^{b}	4.27^{b}	4.50^{c}
M. racemosus + *R. nigricans* (0.1 gL^{-1})	4.78^{ab}	4.88^{ab}	7.30^{a}	7.86^{bc}
M. racemosus (g L^{-1})	6.48^{a}	6.48^{a}	9.07^{a}	11.26^{ab}
R. nigricans (g L^{-1})	5.72^{a}	5.72^{a}	8.40^{a}	9.50^{ab}
M. racemosus (0.1 g L^{-1})	6.60^{a}	6.70^{a}	10.03^{a}	11.10^{ab}
R. nigricans (0.1 g L^{-1})	5.45^{a}	5.45^{a}	7.77^{a}	10.90^{ab}
M. racemosus + *R. nigricans* (g L^{-1}) + Seedplus®	5.33^{a}	5.33^{a}	10.97^{a}	11.33^{ab}
Control	6.75^{a}	8.95^{a}	11.50^{a}	12.50^{a}

*Within each column, means followed by the same letter(s) are not significantly different (P ≤ 0.05) according to Duncan Test. **DAS, Days after sowing.

Table 3. Effects of inoculum density and types of pathogenic mould on radicle length vigour indices of watermelon seeds.

Treatment	Radicle length (cm)				V.I*** (x100)
	5 DAS**	6 DAS	7 DAS	8DAS	8DAS
M. racemosus + *R. nigricans* (gL^{-1})	3.17^{a*}	4.47^{a}	4.57^{a}	4.83^{a}	0.39^{d}
M. racemosus + *R. nigricans* (0.1 gL^{-1})	4.40^{a}	5.90^{ab}	6.37^{ab}	6.87^{ab}	0.89^{c}
M. racemosus (g L^{-1})	5.25^{b}	7.00^{b}	7.40^{b}	7.93^{ab}	1.53^{ab}
R. nigricans (g L^{-1})	4.50^{a}	6.38^{b}	6.47^{ab}	7.23^{a}	1.84^{a}
M. racemosus (0.1 g L^{-1})	5.67^{b}	7.48^{b}	7.96^{b}	8.70^{b}	1.21^{bc}
R. nigricans (0.1 g L^{-1})	6.37^{b}	5.48^{ab}	6.53^{ab}	$7.20^{a\,b}$	1.68^{ab}
M. racemosus + *R. nigricans*(g L^{-1})+ Seedplus®	5.38^{b}	7.38^{b}	8.30^{b}	8.68^{b}	1.60^{ab}
Control	6.38^{b}	7.10^{b}	8.06^{b}	8.70^{b}	1.99^{a}

*Within each column, means followed by the same letter(s) are not significantly different (P ≤ 0.05) according to Duncan Test. **DAS, Days after sowing, ***V.I. = vigour index.

of seeds in the control was the highest (6.75 cm) but was only significantly higher (P≤ 0.05) than for seeds applied with *M. racemosus* + *R. nigricans* (g L^{-1}). At 6 DAS, seeds applied with *M. racemosus* + *R. nigricans* (g L^{-1}) had the least plumule length (3.55 cm) and this was significantly lower (P≤ 0.05) than for all other treatments except those applied with *M. racemosus* + *R. nigricans* (0.1g L^{-1}). At 7 and 8 DAS, seeds in the control had the highest plumule length (11.50 and 12.50 cm, respectively). This was significantly higher (P≤ 0.05) than for seeds applied with *M. racemosus* + *R. nigricans* (g L^{-1}) (Table 3).

At 5 DAS, radicle growth was highest in the control but not significantly different (P≤ 0.05) from fungi inoculated seeds applied with Seedplus®. This was significantly higher (P≤ 0.05) than for seeds applied with *M. racemosus* + *R. nigricans* (g L^{-1}) and *R. nigricans* only (g L^{-1}). At 6 and 7 DAS, radicle length of seeds applied with *M. racemosus* + *R. nigrican s* (g L^{-1}) was significantly lower (P≤ 0.05) than for those applied with *M. racemosus* only (0.1 or g L^{-1}), Seedplus® and the control. At 8 DAS, seeds applied with *M. racemosus* + *R. nigricans* (g L^{-1}) had the least radicle length (4.83 cm) and was significantly lower (P≤ 0.05) than those applied with *M. racemosus* (0.1 g L^{-1}), Seedplus and the control. Seedling vigour index was significantly lower (P≤ 0.05) in seeds applied with *M. racemosus* + *R. nigricans* (g L^{-1}) than for those applied with lower inoculum density (0.1 g L^{-1}). Highest vigour index was obtained in the control (1.99) and this was not significantly different (P≤ 0.05) from the inoculated seeds with the fungi but with Seedplus® treatment.

DISCUSSION

Water melon seeds inoculated with the fungi without fungicide treatment in this study exhibited some pathogenic symptoms such as root rot. Moss and Smith (2006) earlier reported that pathogenic seed-borne fungi include *R. nigricans*, *Mucor* spp. and *Fusarium oxysporum*. Mehrotra and Aggarwal (2003) reported that such fungi could seriously retard seed germination

through softening and necrosis of tissues. They also confirmed the association of seed-borne fungi with seed viability, wilting of plants and stem flaccidity. Incidences of *R. nigricans* and several other pathogenic seed-borne fungi on seeds have been reported by Leslie et al. (2005) and Anjorin et al. (2008). The factors influencing the development of seed-borne fungi include the moisture content of the seed, prevailing temperature, storage period and degree of seed invasion with the pathogen). Others are level of host genetic resistance, activities of insects and mites and amount of foreign materials in the seed lot (Miller and Trenholen, 1994).

The inhibition of radicle and plumule growth especially by seeds applied with high inoculum density led to lower germination percentage of up to 50% (Linn and Ehret, 1991; Gilbert and Tekauz, 1995; Menzies et al., 1996). Pathogenenic fungi may only be present at such low density such that their inoculum potential is low. Thus infected plants may not show significant symptoms of infection even though the pathogen is present in their cell or tissue (Sanogo and Moorman, 1993). Higher inoculum density can overcome the plant's defense mechanisms and cause death. However, the actual level of inoculum needed to overcome the host's defenses would vary with the environmental conditions (Paternotte, 1992). Whether this is because of an increased severity of infection caused by increased number of primary infections at few sites on the roots, or an increased number of infection sites along the roots, is not known. It may be that the seeds applied with low inoculum density are able to overcome the low level of infections, and once the defence responses are activated the plants are able to tolerate the infections that occurred from secondary inoculum produced from primary infections (Menzies et al., 1996). A strong linear relationship between pathogen inoculum density and growth and yield parameters monitored by Sanogo and Moorman (1993) are good indications of how the increasing density of the pathogen inoculum increases the stress on the host plants.

The relatively higher radicle growth observed on the fungal inoculated seeds applied with imidacloprid, metalaxyl and carbendazim (Seedplus®) and also in the control was in line with the submission of Paternotte (1992) on disease development of *Pythium* in glass house cucumber. Seeds treated with Seedplus® recorded relatively high percentage germination. This could be due to the systemic action of Seedplus® which is strongly inhibitory to hyphal development and fungal spore germination (Maynard and Hopkins, 1999). This supports the fact that this seed dressing fungicide was effective in the control of the fungi infection (Freshpatents.com, 2005).

Conclusion

This study confirms that *M. racemosus* and *R. nigricans* were capable of causing considerable low germination and seedling vigour in watermelon. It was also confirmed that the higher the inoculum density especially when the fungi are combined, the greater the pathogenic capacity on watermelon seeds. Thus, infected seeds with low inoculum density had relatively higher germination percentage, plumule and radicle length and seedling vigour than those applied with high inoculum density. Better germination and seedling vigour was observed when only one fungus was involved than when two fungi were involved. Hence inoculum level on seeds should be reduced to the tolerable level through the use of seed dressing fungicide such as imidacloprid, metalaxyl and carbendazim (Seedplus®) before sowing. This could greatly improve radicle and plumule development, germination and vigour indices of watermelon seeds. Farmers should preferably sow watermelon seeds that are clean, fungicide-dressed or fungal-resistant.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Study on the biochemical effects of barley fiber on the hypercholesterolaemic rats

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Chemical compositions and nutritive value of Barley (*Hordeum vulgare)* samples (barley grain and barley hull) were determined by proximate analysis. The percentage value of moisture, ash, protein, fat, fiber, cellulose, lignin and carbohydrate in barley whole grain and hull is deliberated. Moreover, evaluation of functional properties of barley, anti-hypercholesterolaemic effect of barley hull high fiber diet is studied. The in house prepared barley hull high fiber diet proved effective as it lowered the cholesterol levels significantly in hypercholesterolaemic rat.

Key words: Nutritive value, barley and anti hyper cholesterol.

INTRODUCTION

Barley is a cereal grain derived from the annual grass *Hordeum vulgare*. It serves as a major animal feed crop, with smaller amounts used for malting (mostly for beer and certain distilled beverages) and in health food. Barley is an important cereal food all over the world. It is abundantly used in Africa, Asia and semi-arid tropics and is also cultivated in Europe, America and Australia (Keogh et al., 2003; Erkan et al., 2006).

Barley is the second most important winter cereal in Pakistan. It grows on an area of 107.7 thousand hectares with production of 99.6 thousand tones. The average yield is 925 kg per hectare (Government of Pakistan, 2008). About two third of the area devoted to barley in Pakistan is rained and one third is irrigated.

Barley contains many nutrients, including dietary fiber, antioxidants, vitamins, minerals (calcium, magnesium, potassium, phosphorus) sphingolipids and unsaturated fatty acids. This diverse composition allows barley products to have myriad of benefits and appealing characteristics (Oscarsson et al., 1996). Barley is a nutritious cereal grain that offers consumers many bioactive compounds that can help improve their health. The barley is also used as neutraceutical ingredient because it contains high content of soluble fiber, espe-cially as a rich source of β- glucan. Because of its nutritional and chemical properties in particular a high dietary fiber component, barley is considered the as most suitable grain in human diet.

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Many agencies also encourage an increase fiber intake. Barley grain is an excellent source of both soluble and insoluble dietary fiber (DF) with clinically proven health benefits. Beta Glucan (BG) the major fiber constituents in barley have been shown to lower the plasma cholesterol, reduce glycemic index and reduce the risk of colon cancer (Erkan el al., 2006). Other more general beneficial physiological effects of consumption of whole grains include reduced transit time for foods, which may reduce risk of colon cancer (Bruce et al., 2000), and reduced absorption of nutrients which may reduce glucose and insulin responses and risk of obesity (Wisker et al., 1994, 1992).

The purpose of the present work was to investigate the biochemical composition of barley and to estimate hypocholesterolaemic effect of high fiber diet on hypercholesterolaemic rats. We also aimed to prove hypothesis that it reduces cardiovascular disease risk comparatively with other sources of soluble fiber.

MATERIALS AND METHODS

Procurement and cleaning of raw material

Barley whole grain used in this study was purchased from local market. Barley grain and hull was cleaned manually. After removal of foreign material, weeds and non-grain matters, samples were stored in clean polythene bags until used.

Estimation of Moisture, Ash Fat and Fiber

The moisture, ^{A}sh and fat contents were determined by using method of (AOAC, 2012). 3 to 5 g of dried sample was in use in each assessment.

Estimation of protein

Protein content of barley and hull sample was estimated by using Kjeldahl method as reported by AOAC, 2012

Estimation of lignin

Lignin content was estimated by the method of A.S.T.M (1961). 1 g of defatted sample was used for reflux with 70 mL 1.25% H_2SO_4 for 2 h. Reflux sample washed with hot water and then with chloroform. Washed sample treated was with 72% H_2SO_4 for four hours with constant stirring. Sample was filtered (add 10 -12 mL distilled water) with Whatman filter paper No-1 and ignited it at 550°C for 4 h.

Estimation of cellulose

Cellulose estimated by using the method of Kurschner and Hanak (1930). Missing) 1 g of defatted sample was used for reflux with 15 mL of 80% acetic acid and 1.5 mL conc. HNO_3 acid for 2 h. Reflux sample washed with hot water. Washed sample was treated with 72% H_2SO_4 for four hours with constant stirring. Sample was filtered (add 10 to 12 mL distilled water) with Whatman filter paper No. 1 washed with distilled water until it become neutral and finally it washed with alcohol and ignited it at 550°C for 6 h.

Estimation of carbohydrates

The carbohydrates were determined by applying following equation. Moister + Ash + Fat + Fiber + Protein - 100 = CHO.

1) Development of high fiber diet

The complete dehulling of barley hull from barley groats carried out. Barley hull was used as fiber source in diet. After grinding of barley hull, fiber was extracted by hydrolysis. Acid hydrolysis of extract was carried out with dilute sulphuric acid. After hydrolysis, fiber was neutralized by NaOH. Sample was washed with the distilled water for the complete removal of NaOH.

2) Composition of diet

Twenty five (25 g/kg) barley bran high fiber diet contained 175 g/kg casein protein to fulfill the protein requirements in animal body. After that 12 g/kg corn oil was added in diet to provide essential fatty acids. Corn starch and sucrose was added in diet as sweetness and carbohydrates source. DL- Methionine added in diet for amino acid source. Vitamin mixture and mineral mixer was added 10 and 35 g/kg diet, respectively. Barley bran was used as a fiber source and 257 g barley bran was added in per kg diet (Remi et al., 1992). After manually mixing, diet was pelleted with water and dried in oven and store in air tight bags.

Feed intake

Feed intake was determined by the weighing the feed hoppers. Consumption, expressed as grams per rat per day, was obtained by dividing food intake by the number of rats per cage (Jackson et al., 1994).The average feed in take was 13 g/day/rat.

Functional properties of barley bran high fiber diet

High fiber barley hull diet was used for the determination of functional properties of barley by the determination of antihypercholesterolemic effect of high fiber barley hull diet on hypercholesterolaemic rat.

Experimental design and induction of hypercholesterolemia

Thirty (30) albino rats of either sex weighing between 200 to 300 g were selected for the experiment. The rat was divided into 3 groups each group consist of 10 rats.

Induction of Hypercholesterolemia

The rat was made Hypercholesterolaemic by oral administration of 1% of cholesterol powder (1 g/kg) for 10 days as prescribed by (Reeves et al., 1993). Then, the hypercholesterolaemic condition was confirmed by using respective diagnostic kit at the 11th day of experiment. After confirmation of hypercholesterolaemic condition of rats, the day at which rats were treated with 25% barley bran high fiber diet was considered as first day of experiment.

Table 1. Proximate analysis of whole grain barley and hull of barley.

Parameter	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Fiber (%)	Cellulose (%)	Lignin (%)	Carbohydrate (%)
Barley whole grain	11.5 ± 0.38	2.5 ± 0.30	12.5 ± 0.26	1.8 ± 0.15	4.4 ± 0.21	6.6 ± 0.26	0.6 ± 0.21	67.30
Barley hull	6.43 ± 0.25	4.13 ± 0.15	9.2 ± 0.15	1.23 ± 0.20	26.3 ± 0.85	37.5 ± 0.3	2.67 ± .0.25	52.71

The mean value was calculated by applying standard deviation.

Blood collection and analysis

One (1) ml blood was collected from coccygeal vein of albino rats. Collected blood was centrifuged at 3000 rpm for 10 min and serum was separated. The enzymatic kit was used to assess serum levels using spectrophotometer.

Examination of serum cholesterol

The cholesterol level was determined by using method of (Richmond, 1973).

Estimation of cholesterol by enzymatic kit

Twelve (12) test tube were used, two of them labeled as blank/standard. Remaining 10 tubes were labeled as 1, 2, 3……10 for each sample of rat's serum from each hypercholesterolemic group. 1000 µl reagent was taken in all the tubes by micro pipette. 10 µl of standard solution from kit was added to the tube labeled as standard and 10 µl of serum sample was taken in tubes labeled as 1, 2, 3……12. Contents of all the tubes were mixed well and then incubated at 37°C for 10 min. Absorbance of standard and sample was measured against the blank at wavelength of 546 nm. Similar procedure was used the rest of the samples.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using Co Stat-2003 software following the method as described by (Steel and Torrie, 1982). The Duncan multiple range (DMR) was used to determine the level of significance between samples.

RESULTS

The values of moisture, fat, ash, protein, fiber, carbohydrates, lignin and cellulose in whole grain barley and hull of barley are given in Table 1. The moisture content (11.5±0.38) and fat content (1.8±0.15) of whole grain barley was higher than the moisture content (6.43±0.25) and fat content (1.23±0.2%) of hull of barley. The whole grain barley had lower ash contents (2.5±0.30%) as compared to hull of barley (4.13±0.1%).The increased value of crude protein in whole grain barley was12.5 ± 0.26%. In addition to it was observed that the percentage fiber content of whole grain barley was lower than the fiber content of hull of barley.

Contrary, carbohydrate value in whole grain barley was higher than the hull of barley (67.3 and 52.71%) respectively. Proximate composition of the whole grain barley and hull of barley showed that the crude lignin of whole grain barley was lower than the hull of barley. Also the cellulose contents in whole grain barley were lower as compared to hull of barley.

Cholesterol level (mg/dl)

In group A (control) the mean value of cholesterol level at day 0, 1, 14 and 28 of the experiment were 157 mg/dl, 159 mg/dl, 158 mg/dl and 159 mg/dl respectively. Slight variation in cholesterol level from day 1st to 28 was observed which was insignificant.

DISCUSSION

Most research studies have shown the use of barley and barley product as a source of soluble fiber (Dongowski et al., 2002). Barley provides a potential source of low cost protein with good nutritional assessment. The use of Barley in human diet has also been motivated by the quality of its fiber components which act as hypoglycemic and hypochloesterolemic agents. Barley was selected as cereal source because it is rich in important fiber components. The barley contains substantially higher amounts of functional ingredient β-glucan. The use of β-glucan extracted from barley as human food due to its, positive role in human health has received a growing attention (Carpita, 1996).

Our results of proximate analysis of barley sample (barley whole grain and barley hull) and hypocholesterolaemic effect of barley high fiber diet are given in Table 2, which, correlated the earlier findings for barley varieties by (Keogh et al., 2003).

The values of moisture, fat, ash, protein, fiber, carbohydrates, lignin and cellulose in whole grain barley and hull of barley are given in Table 3. The moisture content (11.5±0.38) and fat content (1.8±0.15) of whole grain barley was higher than the moisture content (6.43±0.25) and fat content (1.23±0.2%) of hull of barley (Anderson et al., 1984). The whole grain barley had lower ash contents (2.5±0.30%) as compared to hull of barley (4.13±0.1%),these results are in line with earlier findings of Bridges et al 1992, who have also observed that same combination in case of oat and barley. Increase value of

Table 2. Cholesterol level (mg/dl).

Group	Cholesterol level 0 Day (before Hypocholesterolaemia) (Mean ± S.D)	Cholesterol level 1 Day (after Hypocholesterolaemia) (Mean ± S.D)	Cholesterol level 14 Day (after Treatment) (Mean ± S.D)	Cholesterol level 28 Day (after Treatment) (Mean ± S.D)
Group A: Control	157 ± 4.42	159 ±4.31	158 ± 4.13	159 ± 4.85
Group B: hypocholesterolaemic rats + chick starter diet	162 ± 6.89	220 ± 3.70	198 ± 1.19	174± 1.58
Group C: hypocholesterolaemic rats + high fiber diet	211± 5.10	223 ± 5.73	179 ± 7.30	167 ± 6.70

Table 3. Multiple comparisons of cholesterol level (mg/dl) among different groups.

Dependant variable	Group	Group	Mean difference	Std. Error	Significant
Cholesterol level day 1	Group A Control	Group B: hypocholesterolaemic + chick starter diet	-61.6000	2.08487	0.000*
		Group C: hypocholesterolaemic + high fiber diet	-64.6000	2.08487	0.000*
	Group B hypocholesterolaemic + chick starter diet	Group A: Control	61.6000	2.08487	0.000*
		Group C: hypocholesterolaemic +diet	-3.0000	2.08487	0.162**
	Group C hypocholesterolaemic +diet	Group A: Control	64.6000	2.08487	.000 *
		Group B: hypocholesterolaemic + chick starter diet	3.0000	2.08487	.162**
Cholesterol level day 14	Group A Control	Group B: hypocholesterolaemic + chick starter diet	-67.1000	2.18920	.000*
		Group C: hypocholesterolaemic +diet	-21.3000	2.18920	.000*
	Group B hypocholesterolaemic + chick starter diet	Group A: Control	67.1000	2.18920	.000*
		Group C: hypocholesterolaemic +diet	45.8000	2.18920	.000*
	Group C hypocholesterolaemic +diet	Group A: Control	21.3000	2.18920	.000*
		Group B: hypocholesterolaemic + chick starter diet	-45.8000	2.18920	.000*
Cholesterol level at day 28	Group A Control	Group B: hypocholesterolaemic + chick starter diet	-59.2000	2.17647	.000*

Table 3. Contd.

	Group C: hypocholesterolaemic +diet	-8.6000	2.17647	0.07**
Group hypocholesterolaemic + chick starter diet	Group A: Control	59.2000	2.17647	0.000*
	Group C: hypocholesterolaemic + high fiber diet	50.6000	2.17647	0.000*
Group C hypocholesterolaemic +diet	Group A: Control	8.6000	2.17647	0.001*
	Group B: hypocholesterolaemic + chick starter diet	-50.6000	2.17647	0.000*

crude protein in whole grain barley was12.5 ± 0.26%, similar findings of protein content were estimated by Jackson (1994) in same cereals. In addition to it was observed that the percentage fiber content of whole grain barley was lower than the fiber content of hull of barley. Significant increase in fiber content in hull of barley was reported by Gorinstein et al. (2002). Barley grain is excellent source of soluble and insoluble dietary fiber.

The cellulose content of whole grain barley and hull of barley is given in Table 1. The cellulose and lignin content of whole grain barley was than hull of barley. These results are comparable with those reported by Jacobs et al. (1998).

The second part of this research was aimed to estimate the cholesterol lowering effect of high fiber diet. It was inferred from the outcomes of the present study that the barley high fiber significantly lowers the cholesterol level in hypercholesterolaemic rats.

In this experiment blood analysis was conducted to estimate the cholesterol lowering effect of high fiber diet of barley on hypercholesterolaemic rat. Significant reduction in cholesterol was observed in hypercholesterolaemic group C hypercholesterolaemic high fiber diet as compared to hypercholesterolaemic control.

Significant reduction in cholesterol was observed in hypercholesterolaemic group C hypercholesterolaemic high fiber diet) as compared to hypercholesterolaemic control group.

Barley dietary fiber is high in β-glucan, which helps to lower cholesterol by binding to bile acids and removing them from the body via the feces. Bile acids are compounds used to digest fat that are produced by the liver from cholesterol (Behall et al., 2004).

When they are excreted along with barley fiber, the liver must produce new bile acids and uses up more cholesterol, thus lowering the amount of cholesterol in circulation. Soluble fiber may also minimize the amount of cholesterol prepared in liver (Bazzano et al., 2003).

Conflict of Interests

The author(s) have not declared any conflict of interests.

Conclusion

It can be concluded from the present study that the highest soluble fiber intake had the greatest effect on total cholesterol. The outcomes of present study indicate that the addition of barley to a healthy diet can reduce risk of cardiovascular disease by reducing cholesterol level in hypercholesterolaemic conditions.

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Review

Noni: A new medicinal plant for the tropics

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Noni (*Morinda citrifolia* L.) is a tropical plant belonging to the family, Rubiaceae. It is a small evergreen tree or shrub growing to a height of 3 to 6 m. Fruits are multiple, oblong, 5 to 7 cm long, soft, watery with a cheesy aroma. Fruits contain more than 150 compounds having nutraceutical properties. The major compounds isolated are scopoletine, octaanoic acid, vitamin C, terpenoids, alkaloids, anthraquinones, beta sitosterol, carotene, vitamin A, amino acids, acubin, etc. Xeronine is a miracle ingredient responsible for all therapeutic properties of noni and it is reported to exist in noni as its precursor prexeronine. Noni is most important botanical and dietary supplement traded in international market. The fruit is marketed in different forms such as fermented and pasteurized noni juice, noni powder, noni capsules, etc. Noni is a valuable medicinal plant and it may prove to be one of the more diversely valuable agents in nature's medicine chest, and an enduring dietary supplement which serves the health needs of our country. Based on its ethnobotany, phytochemistry, production, processing and value addition as a highly nutritious medicinal plant, noni is discussed in detail.

Key words: Noni, medicinal plant, nutritious, fermented, pasteurized.

INTRODUCTION

Noni (Morinda citrifolia) is a small tree belonging to the coffee family, Rubiaceae. It is also known as Indian mulberry, awl tree, cheese fruit, nino, nona, etc. For over 2000 years or more, the plant has been identified as a medicinal plant by Polynesians and Tahitians and they used it for therapeutic purposes. Almost all parts of the plant are used for one or other purposes. The fruits were used in food preparations and as medicine. Different parts of the plant such as leaves, stem and roots were also used as medicine. In Polynesia and Southeast Asia, application of plant is extended to cure cough, cold, pain, liver disease, malaria and blood pressure (Dixon et al., 1999). Considering the medicinal value of the plant, National Medicinal Plant Board has included noni in the list of plants approved for cultivation.

ORIGIN AND DISTRIBUTION

The plant is believed to have originated from South-east Asia, Oceania and tropical Australia, extending from Polynesia to India. It is now grown throughout the tropics and is cultivated on a commercial scale in Latin America, from Mexico to Columbia and Venizuela, including Costa Rica, Panama, Kenya, Florida and the West Indies (Abbott and Shimazu, 1985). In India, the plant is growing

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wild in the coastal areas of Kerala, Karnataka, Tamil Nadu, and Orissa. The highest naturally grown populations are seen in Andaman and Nicobar Islands.

BOTANICAL DESCRIPTION

The genus name '*Morinda*' was derived from two Latin words *morus* means mulberry and *indicus* means India, in reference to similarity of fruit of noni to that of true mulberry (*Morus alba*). The species name indicates resemblance of plant foliage with that of citrus species. Noni is evergreen tree or shrub growing to a height of 3-6 m. Noni has a rooting habit similar to that of coffee and citrus with an extensive lateral root system and deep tap root.

Leaves are opposite pinnately reticulate veined, and glossy. Blades are membranous, elliptic to elliptic-ovate, 20 to 45 cm long, 7 to 25 cm long and glabrous with prominent veins. Petioles are stout, 1.5 to 2 cm long. Flowers are perfect; funnel shaped, grouped in globose head or in small clusters, at the leaf axis. The corolla is white, 5-lobed with greenish white corolla tube, 7 to 9 mm long. The flowers give off a sweet fragrance. *Morinda citrifolia* fruits several times annually, producing multiple or aggregate fruits, oblong 5 to 7 cm long with circular scars, which are green when unripe and yellowish-white when fully ripe. The fruits have a soft, watery flesh, and a cheesy aroma which becomes increasingly pronounced and pungent during the ripening process. It is also called starvation fruit.

Noni seeds are small, 4 mm long, reddish-brown, oblong-triangular, and have a conspicuous air chamber. They are buoyant and hydrophobic due to this air chamber and their durable, water-repellent, fibrous seed coat. The seed coat is very tough, relatively thick, and covered with cellophane-like parchment layers. A single large noni fruit can contain well over 100 to 150 seeds. The seeds are edible when roasted.

SOIL AND CLIMATIC CONDITIONS

Noni is an unusual plant, because it can easily tolerate and thrive in a wide range of soils and conditions. The plant is highly tolerant to acidic, saline and alkaline soil conditions and grows well from a pH range of 4.4 to 9. In Hawaii, it can grow under almost any soil conditions at low altitudes. Heavy, compact soils and flood prone areas should be avoided for noni cultivation.

Noni can be grown in wide climatic conditions such as tropical, subtropical, dry and humid climates. It grows naturally in wet to moderately wet conditions, also grown in brackish water. It comes up very well between 20-38°C temperatures. It can be grown from sea level to 2000 m above mean sea level.

HARVESTING AND HANDLING

Under favourable growing conditions, noni plants may begin to produce small flowers and fruits about 9 months to 1 year of age. Fruits can be harvested at this early stage, although they are generally small and few. Some farmers choose to forego harvest during the first or second years in favour of pruning back the branches instead. In Hawaii, noni fruits are harvested year round, although there are seasonal trends in the amount of flowering and fruit production that may be affected or modified by the weather and by fertilizer and irrigation. Fruit production may diminish somewhat during the winter months in Hawaii. A given noni field is usually harvested from two to three times per month.

Noni fruits can be picked at any stage of development, depending on the intended processing method. Some producers prefer green fruits, whereas other processors prefer the hard white noni fruits for processing. Most noni juice processors accept or prefer the "hard white" stage of fruit development for noni juice production, because the fruits ripen quickly once that stage of development is reached.

Noni fruits are harvested by hand by picking the individual fruits from the branches. They are placed in baskets or bags or placed in bins for transport to the processing facility. Noni fruits do not bruise or damage easily, and usually no special padded containers or other precautions are needed to prevent fruit significant fruit damage. Green or unripe yellow noni fruits are very hard-skinned and durable, and therefore resistant to superficial damage and bruising during shipping and handling. Noni fruits at this stage of development will ripen overnight or in a few days at room temperature and can be processed for juice immediately thereafter. Furthermore, exposure of noni fruits to direct sunlight or to warm temperatures immediately after harvest is not a significant concern. So, noni fruits need not be refrigerated after harvest and are usually not refrigerated.

PROCESSING OF NONI

Traditional noni juice

Freshly picked noni fruits after washing are allowed to air-dry on raised tables before they are processed for juice. The most efficient noni juice extraction by weight is obtained when ripe, soft, translucent fruits are placed into the juice collection vessels. Ripe noni fruits are placed into a juice collection vessel for two months or longer. During this time, the noni juice separates (drips) gradually from the pulp. The juice collection and fermentation vessels should be made of glass, stainless steel or food-grade plastic. The noni juice collects inside the containers and ferments as it gradually seeps and sweats from the fruits. The juice appearance is initially an amber or

golden colored liquid that gradually darkens with age. After the collection and fermentation process is complete, the juice is drained from spigots at the base of containers (and filtered). Fresh air is excluded from these containers, and contact between the juice and fresh air is minimized throughout the process. The final noni juice product is decanted, filtered and bottled.

Extraction efficiency (traditional method, drip extraction)

After approximately 2 months, most of the noni juice separates naturally from the fruit pulp and may be drained from the container and filtered. The recovery of juice by this traditional method is approximately 40-50% of the original fruit weight. Therefore, using this method, 100 pounds of fruit may yield about 40-50 pounds of juice, or about 4.5 to 5.0 gallons of juice.

After all the noni juice is drained from the collection and fermentation vessel, the residual pulp may be pressed to express the remaining juice fluids. The leftover pulp and seeds may be discarded, or they may be dehydrated and used in other noni products.

Non-traditional noni juice (fresh-squeezed, filtered and non-fermented)

Fresh-squeezed noni juice has a sweeter (less acidic), fruitier flavor than aged, fermented noni juice. When noni fruits are ripe, the juice is separated from the pulp and seeds using a fruit press. Up to 65% juice recovery by weight is possible using this method of juice extraction. Home producers of noni juice use a wide range of fruit pressing methods, from squeezing by hand through cheesecloth, paint strainers, to more elaborate home-made pressing devices

A hydraulic fruit press is used for making fresh-squeezed noni juice. Ripe fruits are loaded into the press through the top door. Juice is pressed from the pulp and bottled immediately.

Fresh-squeezed noni juice has a golden amber color and has significantly less sediment than fermented noni juice collected by the traditional method. Conversely, fermented noni juice produced by the traditional method is very dark brown, resembling the colour and texture of soy sauce.

Fermentation of fresh-squeezed juice can be arrested by refrigeration or by pasteurization. This will preserve the fruity, sweet taste of the non-fermented juice. Or, the fresh-squeezed juice may be allowed to ferment naturally in bottles or containers for a period of weeks or months prior to marketing or consumption.

Amended or mixed noni juice products

Noni juice may be mixed with other juices of flavorings to improve the palatability of the product (e.g., with raspberry, strawberry or grape flavors). Some producers choose to dilute their juice with water and sweeten the product with the addition of sugar.

In Hawaii, noni fruit pulp may be chopped, dehydrated and powdered and used in reconstituted noni juice products for the dietary supplement industry. These products are standardized to approximately 0.8% noni active polysaccharides (which is equivalent to the noni active polysaccharide content of pure aged noni juice).

Noni juice itself may be evaporated and formed into a powder that can be used in various products, including reconstituted juices. Noni juice powder is highly hygroscopic (attracted to water) and must be mixed with a non-hygroscopic carrier to enable it to retain its powdered formulation.

CHEMICAL CONSTITUENTS OF NONI

Table 1 contains a partial list of chemical constituents isolated from noni, and their proposed or documented medicinal qualities or cultural significance.

Activity of ripe noni fruit and its constituents

The following is a partial list of the phytochemical constituents in ripe noni fruit, and some of their known biological activities.

Xeronine

Xeronine is a miracle ingredient responsible for all therapeutic properties of noni and it is reported to exist in noni as its precursor prexeronine. Xeronine is an alkaloid almost impossible to detect. No independent laboratory has identified or quantified xeronine in any noni product (Heinicke, 1999).

MEDICINAL USES OF NONI

Noni fruit juice concentrates potently activate Cannabinoid 2 which exerts beneficial immunomodulation effects on human body (Palu et al., 2008). Noni extracts demonstrated hypotensive activity, and have been shown to have ACE-inhibitory activity. Since ACE inhibitors are commonly prescribed to treat high BP, this activity points to a potential therapeutic use. It is traditionally used in Malaysia for the treatment of diabetes mellitus. The aqueous extract of noni fruit appears to contain plant compounds that have substantial hypoglycemic property (Jin, 2007). A polysaccharide-rich substance from the fruit juice of noni, noni-ppt is responsible for this activity. It showed synergistic and additive effects when combined

Table 1. A partial list of chemical constituents isolated from noni, and their proposed or documented medicinal qualities or cultural significance.

Noni plant organ(s)	Compound(s)	Proposed or documented effects of compounds
Fruit and fruit juice	Alkaloids (xeronine)	In theory, xeronine enhances enzyme activity and protein structure
	Polysaccharides (glucuronic acid; galactose; arabinose; rhamose; glycosides; trisaccharide fatty acid ester)	Immuno-stimulatory; immuno-modulatory; anti-bacterial; anti-tumor; anti-cancer.
	Scopoletin	Dilates vasculature and lowers blood pressure; anti-bacterial and anti-fungal; anti-inflammatory; analgesic; histamine-inhibiting; arthritic conditions; allergies; sleep disorders; migraine headaches; depression; Alzheimer's disease.
	Vitamins and Minerals: magnesium; iron; potassium; selenium; zinc; copper; sulfur; ascorbic acid (vitamin C).	The positive medical effects of the vitamins and minerals in noni juice are well documented. For any questions or medical and health concerns, consult a physician or dietician.
Foliage and cell suspensions	Anthraquinones (damnacanthal)	Antiseptic and antibacterial effects in digestive tract (*Staphylococcus, Shingela, Salmonella*).
	Glycosides (flavonol glycoside; iridoid glycoside, "citrifolinoside")	Anti-cancer effects: (DPPH free radical scavenging activity; inhibition of UVB-induced Activator Protein-1 activity in cell cultures.
Roots	Anthraquinones (damnacanthal)	Inhibits formation of lung carcinoma in mice.
	Morindin and Morindone	Dyes, yellow and red colorants used for tapa cloth; anti-bacterial.

Source: http://www.noni.org/.

with broad spectrum of chemotherapeutic drugs (Hirazumi and Furusawa, 2003). The alcoholic extracts of noni fruits is known to have analgesic effect and it is commonly used for opened sores and inflamed areas of the skin in multiple ethnic groups (Punjanon and Nandasri, 2005).

Ripe noni fruit contains a concentration of anthraquinones including one called damnacanthal, which possess purgative activity. This accounts for the "cleansing" effect described by many users. In the cases of sluggish digestion and slow moving bowels, noni can exert a stimulating and thereby beneficial effect, helping to increase peristalsis and cleanse the colon. Anecdotal accounts of anti-inflammatory effects resulting from noni fruit consumption are too numerous to dismiss. The anti-inflammatory effects of asperuloside, eugenol and scopoletin present in ripe noni fruit would support such a claim. Other agents may possess additional anti-inflammatory activity for the aging population (Amy and Noelani, 2009).

In China, Japan, and Tahiti, various parts of the tree serve as tonics and to treay fever, eye and skin problems, gum and throat problems as well as constipation, stomach pain, or respiratory difficulties. In Malaysia, heated noni leaves applied to the chest are believed to relieve coughs, nausea, or colic. The fruit is used as a shampoo in Malaysia, where it is said to be helpful against head lice. The noni fruit is taken, in Indochina especially, for asthma, lumbago and dysentery. As for external uses, unripe fruits can be pounded, then mixed with salt and applied to cut or broken bones. In Hawaii, ripe fruits are applied to draw out pus from an infected boil. The green fruit, leaves and the root/rhizome have been used to treat menstrual cramps and irregularities. Noni seed oil is abundant in linoleic acid that may have useful properties when applied topically on skin, for example, anti-inflammation, moisture retention.

MARKETING OF NONI FRUITS

Noni is distributed in more than 50 countries across the globe. Its health benefits have been realized by millions of consumers. Noni does not require large amount of fertilizers and irrigation and it grows even in drought prone areas. As all parts of noni are marketed as different products, marketing opportunities have been increased. Over 200 companies are selling noni products. Noni bears 1.5-2 q of fruits/plant/year. The profit is about Rs.2.25 lakhs/acre/year. Even if the yield is 50% of the expected yield, overall the noni can be a blessing for the farmers of all categories and having any type of land.

Noni is most important botanical and dietary supplement traded in international market. The fruit is marketed in different forms such as fermented and pasteurized noni juice, noni powder, noni capsules, etc. Now it is widely available in health food stores, pharmacies, grocery stores and also through the online markets. Noni juice was number one in 2005, for sales of single herbs in the USA, with sales estimated at about $250 million (http://www.ctahr.hawaii.edu/noni/).

Conflict of Interests

The author(s) have not declared any conflict of interests.

CONCLUSION

Considering the positive discoveries made with noni fruit thus far, there is good reason to anticipate that further studies will prove the fruit and its preparations beneficial to health in numerous ways. Noni is a valuable medicinal plant. And it is likely to become an increasingly sought-after dietary supplement. Noni may prove to be one of the more diversely valuable agents in nature's medicine chest, and an enduring dietary supplement which serves the health needs of many.

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In vitro and *in vivo* production of polygalacturonase, polymethylgalacturonase and cellulase enzymes by *Alternaria solani* at different incubation periods

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The production of polygalacturonase (PG), polymethylgalacturonase (PMG) and cellulase (Cx) enzymes have been studied at different incubation period. The fruit rot pathogen of tomato *Alternaria solani* was cultured at 28°C on semi ripe tomato fruit broth medium for 2, 4, 6, 8, 10 and 12 days. Isolated pathogen *A. solani* has a capability of producing PG, PMG and Cx enzyme within short incubation period, that is, 2 days incubation. Production of these enzymes gradually increased by increasing the length of incubation period up to 6 days and further increase in length of incubation up to 12 days, did not show any effect on the production of PG, PMG and Cx. The 6 days incubation was found to be the best incubation period for the production of all these three enzymes. The production of PG and Cx was also more in comparison with PMG enzyme. The enzyme activity in healthy and diseased semi ripe tomato fruits was also assayed at different incubation periods and it was found to be maximum in diseased tomato fruits in comparison with healthy tomato fruits. It was also found that PG and PMG enzymes were produced in higher concentration than the Cx enzyme. Among six incubation periods, 6 days incubation period was found best for maximum production of all these three enzymes.

Key words: *Alternaria solani*, semi ripe tomato fruit medium, semi ripe tomato fruit, incubation period.

INTRODUCTION

Fungal diseases of fruit and vegetable plants are known to cause great damages all over the world. Tomato (*Lycopersicon esculentum* Mill.) is the most ancient among the vegetable fruits. Among the fungal diseases, fruit rot is the most severe disease of tomato, which is caused by *Alternaria solani* (Mehta, 1973; Chaurasia, 2001; Chaurasia and Chaurasia, 2010 and Chaurasia et.al., 2013a).

Pectin is present in the middle lamella of cell wall, in the form of magnesium and calcium pectate. They are complex and colloidal in nature and mostly comprises of anhydroglacturonic acid units, linked together by α-1,4

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glycosidic linkages to form a chain like structure of poly-galacturonic acid units. The middle lamella is degraded by pectolytic enzymes secreted by the phytopathogens. However, the production and activity of pectolytic enzymes by fungal pathogens is an important process of pathogenesis (Gupta, 1956; Wood, 1960; Brown, 1965; Bateman and Millar, 1966; Mehrotra et al., 1988).

Next to pectin, the cellulose is the major component and basic unit of structural framework of plant cell walls, which act as barrier. The pathogens feed successfully only after breaking this barrier with the help of cellulolytic enzyme. Cellulolytic enzymes secreted by pathogen play on important role on living plant tissues in softening them and may further participate indirectly by hydrolysing cellulose into soluble saccharides which serve as food for pathogen. The production of cellulolytic enzymes and degradation of cellulose by several fungi has been studied by many workers like Reese (1956, 1963), Basu and Ghose (1960), Gascoigne and Gascoigne (1960), Bateman (1964), Norkrans (1967), Singh and Singh (1988), Kaur et al. (1989) and Sohail et al. (2011). Production of cellulolytic enzymes by several pathogenic fungi and its role in the development of diseases has also been reported (Lucas and Sherwood, 1966; Hasija and Batra, 1982; Sanghi and Rajak, 1987; Singh et al., 1991; Singh and Shukla, 1999; Sharma, 2000; Chaurasia, 2001; Shukla and Dwivedi, 2012; Jat et al., 2013, Chaurasia et al., 2013b, c).

Keeping the above facts in mind, in the present paper, the production and activity of pectolytic and cellulolytic enzymes by *A. solani* in culture and in the healthy and diseased tomato fruits at different incubation periods have been investigated.

MATERIALS AND METHODS

Organism

A. solani (Ellis & Marts) Jones & Grout was isolated from the diseased fruits of tomato (Chaurasia and Chaurasia, 2010). The organism was maintained on potato dextrose agar slants.

Production of enzymes *in vitro*

The semi ripe tomato fruit medium tested by Chaurasia et al. (2013b) was used to study polygalacturonase (PG), polymethyl galacturonase (PMG) and cellulase (Cx) enzyme production *in vitro* at different incubation periods. The pathogen *A. solani* was grown in 150 ml Erlenmeyer flask containing 25 ml of semi ripe tomato fruit medium. The flasks were sterilized at 15 lb/sq in pressure for 15 min. After sterilization, each flask was inoculated by a 8.0 mm disc taken from the periphery of four day old colony of the pathogen growing on potato dextrose agar medium. The inoculated flasks were incubated for 2, 4, 6, 8, 10 and 12 days under stationary conditions at 28°C. Three replicates were taken in each case. After desired incubation, fungal mat was removed from the medium and the culture fluid was diluted with 35 ml of distilled water. The filtrates thus obtained was centrifuged for 15 min at 10,000 rpm and the supernatant was used as crude enzyme extract.

Production of enzymes *in vivo*

Semi ripe tomato fruits were used for this purpose. Healthy semi ripe tomato fruits were first surface sterilized with 0.01% mercuric chloride and then washed several times with sterilized distilled water. The surface sterilized fruits were inoculated with *A. solani* by cavity method (Granger and Hornes, 1924; Chaurasia et al., 2009; Chaurasia and Chaurasia, 2010). The inoculated fruits were kept in sterilized moist chambers having 98-100% relative humidity. These chambers were incubated at 28°C. Healthy surface sterilized fruits were kept as control. After 2, 4, 6, 8, 10 and 12 days of incubation, the infected tissue was taken out with the help of a spatula. Thirty grams of diseased tissue was mixed with 30 ml distilled water and the mixture was homogenized in Waring blender for 10 min. The homogenate thus obtained was centrifused for 20 min at 10,000 rpm. The supernatant was used as enzyme extract. Enzyme extract from uninoculated healthy tissue was also prepared in a similar manner.

Assay of enzyme activity

Enzyme activity was measured by using standard viscometric method (Hancock and Millar, 1965; Capellini, 1966; Chaurasia et.al., 2013c).

Oswald viscometers were clamped in stands which were fixed vertically in water bath, with temperature adjusted to 28°C. For the assaying of PG, PMG and C_X enzymes, the following freshly prepared substrate components were used:

1. Polygalacturonase (PG): 1.2% sodium polypectate - 3.5 ml; distilled water- 1.5 ml; citrate phosphate buffer (pH 4.6) - 1.5 ml;
2. Polymethylgalacturonase (PMG): 1.2% citrus pectin - 3.5 ml; distilled water- 1.5 ml; citrate phosphate buffer (pH4.6) - 1.5 ml.
3. Cellulase (C_X): 1.2% carboxymethyl cellulose- 3.5 ml; distilled water- 1.5 ml; citrate phosphate buffer (pH 5.5) - 1.5 ml.

At the time of determination of PG, PMG and C_X enzyme activity, desired substrate component was taken into the stalk bulb of viscometer. Then, 1.5 ml of freshly prepared enzyme extract was poured into viscometer and then efflux time of the enzyme reaction mixture was determined at the intervals of 0, 20, 40, 60 and 80 min. Efflux time for 8.0 ml of distilled water was also noted in each viscometer.

Determination of percent loss in viscosity

Percent loss in viscosity was calculated with the help of the following formula (Capellini, 1966; Chaurasia et.al. 2013c): where, ET_0 = Efflux time in seconds at zero time/control. ET_t = efflux time in seconds at any specific interval of time. ET_w = efflux time

$$\text{Percent loss in viscosity} = \frac{ET_0 - ET_t}{ET_0 - ET_W} \times 100$$

Table 1. Production of PG, PMG and Cx enzymes by *A. solani* in semi ripe tomato fruit medium at different incubation periods.

Days after incubation	Polygalacturonase (PG)					Polymethylgalacturonase (PMG)					Cellulase (C_X)				
	Enzyme activity (%Loss in viscosity)					Enzyme activity (%Loss in viscosity)					Enzyme activity (%Loss in viscosity)				
	Reaction time (min)					Reaction time (min)					Reaction time (min)				
	20	40	60	80	REA	20	40	60	80	REA	20	40	60	80	REA
2	32.5	43.4	50.3	53.2	65.01	7.2	15.3	19.4	21.2	0.00	42.5	52.3	58.2	62.2	85.03
4	53.4	66.3	76.0	78.3	106.83	9.3	19.3	25.4	30.0	16.93	50.4	62.3	68.2	71.4	100.80
6	56.3	68.2	77.4	80.2	112.61	10.4	22.4	30.2	40.3	20.13	66.6	78.4	84.4	88.3	133.33
8	37.5	47.3	52.1	55.1	75.01	8.2	13.4	16.3	18.3	0.00	31.2	41.3	47.5	50.2	62.42
10	32.3	42.4	48.3	51.4	64.64	8.0	11.3	14.6	16.2	0.00	20.3	29.4	35.4	38.2	29.40
12	28.0	32.2	35.3	37.3	56.02	7.4	9.8	11.2	13.4	0.00	18.0	26.2	31.3	33.6	26.20

in seconds for distilled water.

Determination of relative enzyme activity (REA)

Values for percent loss in viscosity were determined for 0, 20, 40, 60 and 80 min reaction time. These values were then plotted against the reaction time, thus a curve was obtained and from this curve, the time to bring a 25% loss in viscosity was determined. REA was then calculated using the following formula:

$$REA = \frac{1000}{t}$$

Where, t = represent the time in min to reach 25% loss in viscosity. Thus:

$$\mathbf{REA = \frac{1000}{25}}$$

RESULTS AND DISCUSSION

Production of PG, PMGand Cx enzymes *in vitro*

The pathogen *Alternaria solani* was cultured in the semi ripe tomato fruits medium and incubated for 2, 4, 6, 8, 10 and 12 days at 28°C. In obtained culture filtrates, the activity of PG, PMG and Cx enzymes was assayed and results are presented in Table 1 and Figure 1. It is clear that pathogen *A. solani* was able to produce PG, PMG and Cx enzymes with a short period of incubation, that is, within 2 days. The gradual increase in incubation period up to 6 days, the activity of PG, PMG and Cx enzymes significantly increased in the culture filtrates. The 6 day of incubation period was found to be the best for the maximum production of PG, PMG and Cx enzymes as 80.2 (REA 112.61), 40.3 (REA 20.13) and 88.3 (REA 133.33) percent loss in viscosity has been recorded respectively at 80 min of reaction time. After 6 days, further increase in incubation period up to 12 days, has no effect and production of PG, PMG and Cx enzymes declined gradually. Mehta et al. (1974) has reported the maximum production of PG, PMG and Cx enzymes in between 4 to 12 days, in culture filtrates of *A. solani* and *Alternaria tenuis*. Mehta and Mehta (1985) also reported maximum PG, PMG and Cx enzyme activity in 6 days old culture of *Fusarium oxysporium*. The gradual fall in production of PG, PMG and Cx enzymes in long incubation period could be due to the slow inactivation of PG, PMG and Cx enzymes by the appearance of oxidized phenols in the semi ripe tomato fruit medium. The same explanation could hold for the observation of Harter and Weimer (1921) on the production of pectic enzyme by *Rhizopus nigricans* and *Rhizopus artocarpi*, in sweet potato broth. Similar observations were made by Balasubramanian and Srivastava (1973).

Production of PG, PMG and Cx enzymes *in vivo*

Data (Table 2 and Figure 2), indicates the activity of PG, PMG and Cx enzymes in the diseased and healthy tissue of semi ripe tomato fruits, in different incubation periods. From the results, it is evident that PG, PMG and Cx enzymes present in healthy tomato fruits and maximum activity of these enzymes were recorded in 6 days incubition period. After 6 day incubation, with the age, the activity of PG, PMG and Cx enzymes was decreased gradually in healthy tissues. It was also observed that the activity of PG and Cx enzymes has completely disappeared in healthy tomato fruits kept for 12 day incubation.

From the above result, it is concluded that the presence of PG, PMG as well as Cx enzyme in healthy tissues shows their constitutive nature. Comparatively, PG enzyme has been found to be

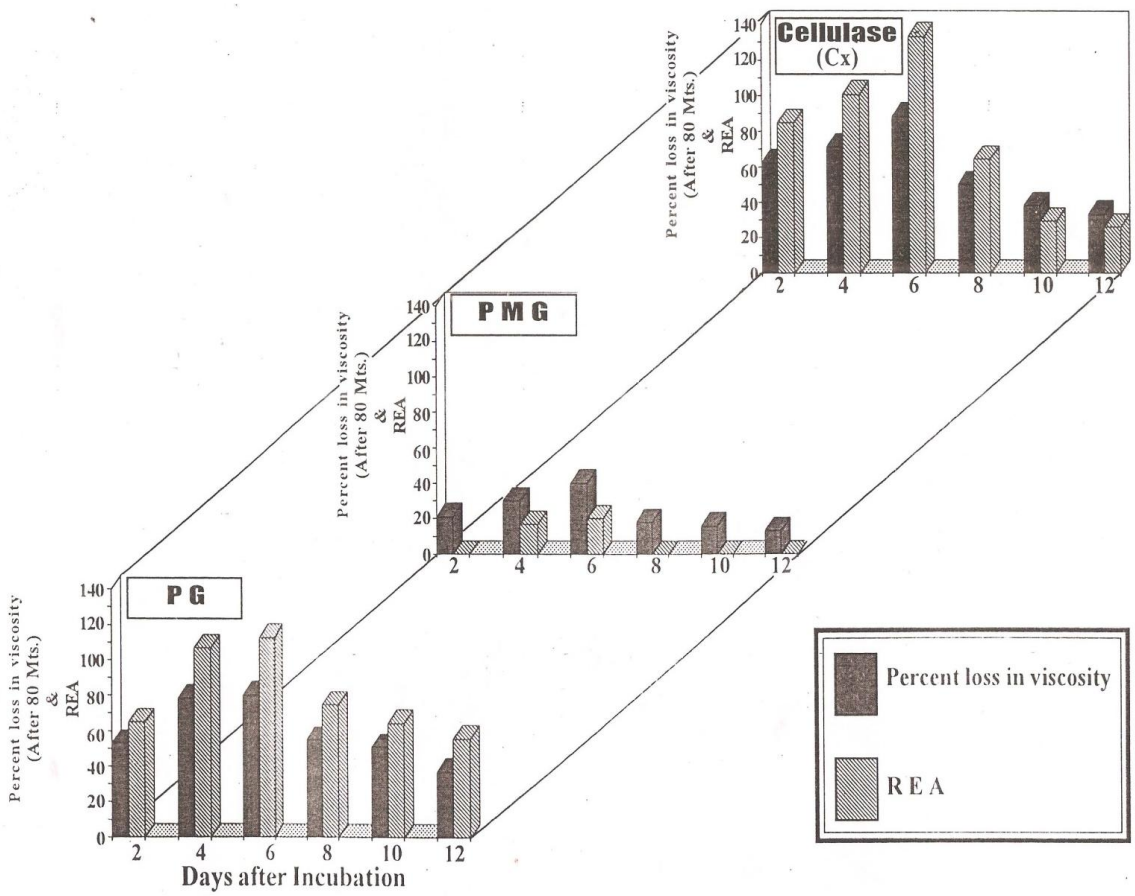

Figure 1. Production of PG, PMG and Cx enzymes by *A. solani* in semi ripe tomato fruit medium at different incubation periods.

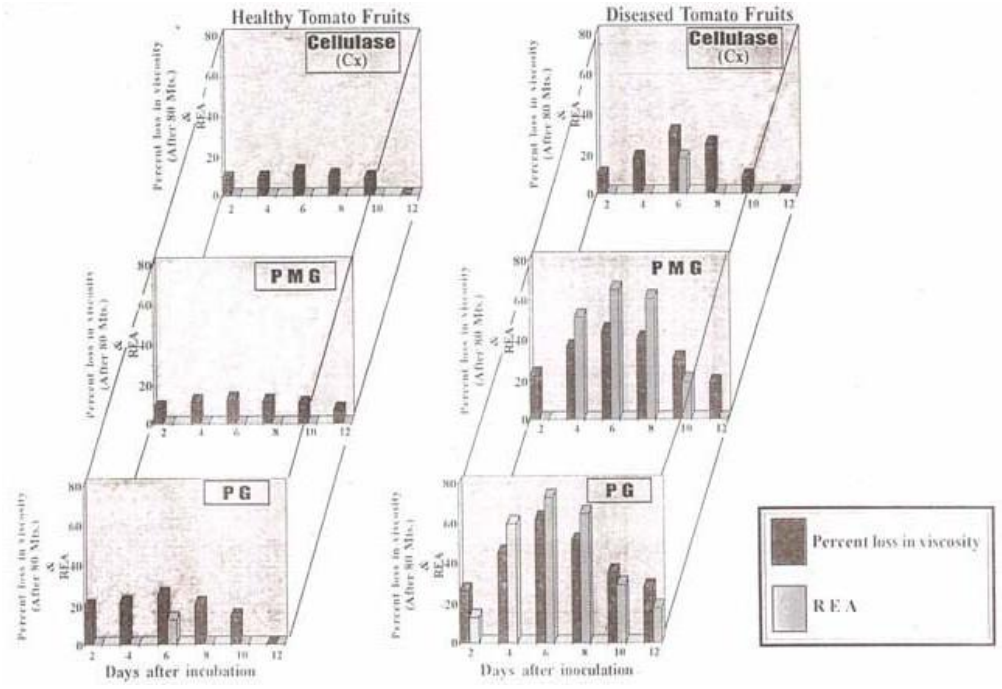

Figure 2. Production of PG, PMG and Cx enzymes in healthy and diseased semi ripe tomato fruits at different incubation periods.

Table 2. Production of PG, PMG and Cx enzymes by *A. solani* in healthy and diseased semi ripe tomato fruits at different incubation periods.

Days after incubation	Polygalacturonase (PG)					Polymethylgalacturonase (PMG)					Cellulase (Cx)				
	Enzyme activity (%Loss in viscosity)					Enzyme activity (%Loss in viscosity)					Enzyme activity (%Loss in viscosity)				
	Reaction time (min)					Reaction time (min)					Reaction time (min)				
	20	40	60	80	REA	20	40	60	80	REA	20	40	60	80	REA
								Healthy tomato fruits							
2	8.00	14.30	18.00	19.00	0.00	6.00	7.00	8.00	8.00	0.00	4.60	6.00	7.50	8.50	0.00
4	8.00	16.00	19.20	21.10	0.00	6.20	8.00	10.00	11.00	0.00	5.00	7.5.	8.60	9.00	0.00
6	8.20	17.40	22.80	25.20	12.60	6.40	8.60	10.20	12.10	0.00	7.20	9.30	11.40	12.00	0.00
8	8.00	15.10	18.30	20.30	0.00	6.10	8.00	10.00	11.20	0.00	7.00	8.00	9.50	10.00	0.00
10	6.20	10.20	13.40	14.00	0.00	5.40	7.20	9.50	10.00	0.00	6.50	7.00	8.00	9.00	0.00
12	0.00	0.00	0.00	0.00	0.00	4.50	6.00	7.00	7.00	0.00	0.00	0.00	0.00	0.00	0.00
Days after inoculation								**Diseased tomato fruits**							
2	16.40	21.40	24.10	26.30	13.15	12.20	17.50	20.80	22.00	0.00	5.40	7.00	8.00	9.00	0.00
4	30.00	39.60	43.30	45.20	7.02	25.60	31.40	34.60	36.10	51.20	6.00	11.30	15.30	17.50	0.00
6	36.50	48.20	57.70	62.20	73.04	32.60	38.30	42.50	44.20	65.23	13.30	21.60	26.50	30.30	17.66
8	32.60	43.30	48.30	51.30	65.23	30.50	35.00	38.30	40.00	61.01	11.70	18.40	22.30	24.60	0.00
10	19.00	29.40	33.40	35.40	29.40	18.60	24.60	28.40	30.30	18.93	4.50	6.50	7.80	8.00	0.00
12	12.40	22.10	26.50	28.10	17.66	9.40	14.00	17.00	18.00	0.00	0.00	0.00	0.00	0.00	0.00

more active in healthy tissues than the PMG and Cx enzyme. Ulrich (1958), Mc Cready et al. (1955) has also reported the higher activity of PG enzyme in healthy tissues of various fruits.

In inoculated diseased semi ripe tomato fruit, the activity of PG, PMG and Cx enzymes were found to be higher in comparison with healthy tissue at various given incubation period. Within a short incubation period, that is, within 2 day, activity of PG, PMG and Cx enzymes has been noted in diseased tissues, which increased with increased in incubation period up to 6 days. The 6 days incubation period was found to be the best for the production of PG, PMG and Cx enzymes in diseased tissues, in which 73.04, 65.23 and 0.0 REA were observed respectively. After 6 day incubation, further increase in incubation period had no effect on the production of PG, PMG and Cx enzymes. In 12 day old infected tissues, the Cx activity has completely disappeared which indicates the inactivation of Cx enzyme in a very long incubation period.

Comparatively, the activity of PG and PMG enzymes were found to be always higher than the Cx enzyme in diseased tissues of various incubition periods, which indicated that PG and PMG enzymes played a significant role in pathogenesis of *A. solani*. To some extent, these results are similar and in agreement with the work of Mehta et al. (1974), Agarwal and Gupta (1978), Hasija and Chawdhury (1979), Sanghi and Rajak (1987) and Sharma (2000).

In general, production of PG, PMG and Cx was higher *in vitro* than *in vivo*. This could be due to the negligible inactivation of these enzymes by phenols present in the semi ripe tomato fruit medium or perhaps the fungus is forced to secrete the enzymes in large quantities to spread in the semi ripe tomato fruit medium. This situation does not prevail when the fungus is grown in host tissue. The ability of the fungus to secrete PG, PMG and Cx *in vitro* and *in vivo* indicates the importance of these enzymes in the pathogenesis of the fungus.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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