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Urea induced urease activity is coupled to the synthesis of primary monoamine: a possible mechanism of enhancing fertilization of mango flower

Md. Shahidul Haque[1*], Uttam Kumar Roy[1*], Farzana Ashrafi Neela[2], Md. Monirul Islam[3] and Md. Faisal Kabir[3]

[1,3]Department of Biochemistry and Molecular Biology, Laboratory of Protein and Enzyme Research, University of Rajshahi, Rajshahi-6205, Bangladesh, [2]Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh

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Mango is a delicious and nutritious fruit however its production is retarded by different ways although the mechanism is not clarified. In this study, we analyzed mango flower and examined the effect of urea on urease activity in flower extract. The flower had 12.0 g of protein, 1.21 g of lipid, 2.65 g of sugar, 0.01 g of reducing sugar, 4.01 g of starch, non reducing sugar 2.64 g in 100 g of flower and the moisture content is 77.15%. The higher urease activity in response to 100 mM urea was found in flower extract rather than 50 and 200 mM concentrations. The K_m and V_{max} were 62.5 mM and 0.04 µmole/mg of protein/min for the above doses respectively. Moreover, 100 mM urea was found to cause a higher synthesis of protein in the flower. Primary monoamine has been recognized to be the fertilization of flower and was found to increase whenever the flower extract was treated with 100 mM urea. Therefore, the results demonstrate that urea is involved in enhancing urease activity which is coupled to the synthesis of protein and the overall bioprocess might be linked to the higher synthesis of primary amine augmenting the higher fertilization of mango flower.

Keywords: mango flower, urease, uptake of NH_4^+, primary monoamine, fertilization of mango flower

Introduction

Mango flowering is a seasonal effect yielding mango fruits and thereby playing the benefit to the economical aspects in the country. Optimum flowering and fruiting are important however change of climate and other environmental factors impairs the flowering and thereby mango production is retarded. Recent investigation reveals that urea fertilization enhances the improvement of various metabolic and physiological aspects in plants and can serve as a rapidly available N-source for plant growth (Wang *et al*., 2008).

Therefore, urea has been recognized to be a potent N fertilizer. The impairment of fertilization in flower might be due to the deficiency of amino nitrogen which is the most limiting element in plant nutrition. Efficient recycling of reduced nitrogen present in the form of urea is important for plant growth since urea contains a significant amount of this element. In addition to internally generated urea, externally applied urea can also be utilized by plants. Urea is a widely used fertilizer because of its low cost, easy in handling and high nitrogen content. In plant, urease is the only enzyme that is able to recapture nitrogen from urea. Many organisms, including plants, some bacteria, fungi and invertebrates, synthesize urease. Ureases are metalloenzymes depend on nickel (Ni^{+2}) in its activity and have been demonstrated to act as catalyzer for breakdown of urea to form two molecules of NH_3 and one of CO_2 (Allison *et al*., 2006; Balasubramanian and Ponnuraj, 2008). The rate of reaction is 8×10^{17} times faster than the reaction without enzyme (Callahan *et al*., 2005). Ureases are widely distributed in plants especially in legume (*Canavalia ensiformis* and *Glycine max*), fungi, and bacteria (Menegassi *et al*., 2008). In spite of presence of urease in high concentrations in seeds of several species of the leguminosae, also found in lower concentrations in the vegetative organs in other families. For uptake of urea in mango flower, urease may play the significant role although the metabolic aspects in flowering are not clarified. Various trials were done to raise fruit set, minimize the percentage of fruit drop, increase tree yield and improve fruit quality by spraying trees with some macro and micronutrients (Ebeed *et al*., 2005). Their findings may augment the general concept that urea may induce the fertility and other characteristics regarding the flowering and fruit development of mango.

Fertilization with urea through leaves could be an efficient method of plant feeding and any modifications leading to increased urease activity in leaves could result in more effective assimilation of this fertilizer. Such an increase might have positive impact on the nitrogen metabolism in plants since more ammonia would be available for assimilation. There is a possibility that the reduced activity of the enzyme may impair the fertilization process. Moreover, recent investigation suggests that deficiency of amino nitrogen causes pathogenic syndromes, pale appearance associated with other physiological disorders in plants (Zhao *et al*., 2005). Plants treated with urea, however increased fruit size and weight, the contents of ascorbic acid, reducing and total sugars in the fruits and also decreased fruit acidity (Ghosh and Chattopadhyay, 1999) which show clearly that urea might be involved in enhancing the flowering, fertility and influencing the cellular metabolic process of mango tree. The disappearance of amino nitrogen from urea is an important aspect in nitrogen metabolism and an essential step for utilization to promote growth.

However, the utilization of amino nitrogen is disrupted in different ways: 1) Deficiency of microorganisms involved in nitrification and denitrification processes; 2) Simultaneous cultivation of crops in the same land; 3) Insufficient photosynthesis. These are associated with impaired N metabolism in plants. Therefore, fixation of nitrogen in plants is an important aspect in nitrogen metabolism.

Primary mono amine has been recognized to be stimulatory factor for fertilization of mango flower and protective functions (Dey and Harborne, 1997). Higher production of mango after flowering might be retarded because of the deficiency of primary monoamine. Therefore, it is assumed that if monoamine concentration is induced by any ways, mango production will be accelerated. As a growth promoting agent and N-fertilizer, urea can induce the synthesis of primary monoamine. Therefore, the current investigation has been designed to clarify the role of urea on the synthesis of primary monoamine in mango flower and may induce the fertilization in this plant.

Material and methods

Plant material

Mango flower (*Magnifera indica*)(Langra) was collected from the mango garden located to the northern side of the University Campus during February-March. The flowers were quickly stored at –80 ^{o}C refrigerature. About 5–6 g of flower were homogenized with mortar and pestle with 10 mM phosphate buffer (pH 7.0) and centrifuged at 6000 rpm for 10 min. After centrifugation, the supernatant was collected and used as crude extract while the sediment was discarded.

Analysis of mango flower

For determination of water soluble protein content from the flower extract, 5–6 g of mango flower were used and analyzed protein following the method of Lowry et al. (1951). Lipid content in flower was determined by the conventional method as described in Laboratory Manual in Biochemistry (Jayaraman, 1981) where 2 g of mango flower were used. Total sugar and reducing sugar content of mango flower was determined colorimetrically as described by Jayaraman (1981). Five to six g of flower were used for this assay. Non reducing sugar or sucrose content was calculated from the following formula:

% sucrose or non reducing sugar = (% of total sugar – % reducing sugar)

The starch content of the mango flower was determined by the anthrone method accordingly as described by Jayaraman (1981) where approximately 5 g of mango flower were used and the moisture content was determined by the conventional procedure in which 2 g of flower were used for this determination.

Assay of urease activity

5.0 g of mango flower were placed into a mortar with pestle and were homogenized with 30 ml of 0.2 M phosphate buffer (pH 7.0) and centrifuged at 6000 rpm for 10 min. The supernatant was collected and used as crude extract. The urease activity in crude extract of flower was assayed by the method as described by Jayaraman (1981). For assay of urease activity, 0.2 or 0.4 ml of the crude extract were used. To examine the effect of urea on urease activity, 50, 100 and 200 mM of urea were used as substrate of the enzyme. The enzyme activity was expressed as µmole/min/mg of protein.

Assay of protein content in flower extract

Flowers (5 g) were homogenized with 10 mM phosphate buffer (pH 7.0) (30 mL) and were centrifuged at 6000 rpm for 10 min. The supernatant from flower homogenate were used as crude extract for assay of protein. 400 µL flower extract, 3.1 mL of phosphate buffer (pH 7.0) and 0.5 mL of 100 mM urea were taken in a test tube and was incubated with 55 oC for 15 min. After incubation, 1 mL 1N H2SO4 was added to the test tube to stop the reaction. For control, 400 µL flower extract, 3.6 mL phosphate buffer (pH 7.0), 1 mL of 1N H2SO4 were mixed in a test tube and was incubated with 55 oC for 15 min. After incubation, the above mixture was cooled. The protein content in 100 □L of the above sample and control was determined by the procedure of Lowry et al. (1951). Briefly, alkaline solution was prepared by mixing 50 ml of alkaline Na2CO3 solution (2% Na2CO3 in 0.1N NaOH) and 1.0 mL of copper-sodium potassium tartarate solution (1 g sodium potassium tartarate and 0.5 g CuSO4. 5H2O were dissolved in 100 mL distilled water). 100 □L of tissue extract was taken to the test tube and made up to 1 mL with distilled water. For blank, 1 mL water was used in place of tissue extract. 5 mL alkaline solution was added to each tube and mixed well. The tubes were allowed to stand for 10 min at room temperature and 0.5 mL of diluted FCR (Commercial FCR was diluted with equal volume of water) was added and mixed well. After 30 min, the absorbance was taken at 650 nm against the blank. The protein content in flower extract was calculated from the standard graph of bovine albumin (1 mg/mL) and is expressed as g/100 g of flower weight.

Test of primary monoamine

400 µL samples (5 g of flower in 30 mL solution) in a test tube was taken, mixed well with 0.5 mL urea (100 mM) and incubated at 55 oC for 15 min. After incubation, 1.0 mL diluted HCl and 4 drops of 10% NaNO2 were added and cooled. In another test tube, a few drops of alkaline β-napthanol were taken and the mixture of the previous test tube was added slowly to the alkaline β-napthanol. An orange deep color was appeared showing the synthesis of primary monoamine in the flower sample. Control tube was similarly used for identification of primary amine where no urea was used and 1.5 mL diluted HCl were used and the color pigmentation was different from the urea induced sample tube.

Results

Analysis of mango flower

To identify the factors involved in fertilization of mango flower, the crude extract from flower was prepared and analyzed. As shown in Table-1**,** the total protein content in the flower extract was 12.0 g while the total lipid content 1.21 g/100 g of flower was observed. The sugar and reducing sugar contents were recorded as 2.65 g and 0.01 g/100 g of flower respectively. The amount of non reducing sugar and starch in the flower extract were 2.64 g and 4.01 g/100 g of the sample respectively. The moisture content in this flower was found to be 77.15%. The results would indicate that the mango flower is a good source of protein and other essential molecules; however, the amount of protein was found to be higher and may play the role in essential biological functions.

Effect of different doses of urea on urease activity

Urea is a potent N-fertilizer inducing the growth of plant and is involved in inducing diverse metabolic and physiological functions. To analyze whether urea is involved in stimulation of urease activity in flower of mango, different doses of urea (50, 100 and 200 mM) were used as substrate for the enzyme. The urease activity in 200 µL flower extract in response to 50 mM urea was 0.0250 µmole while for the control, the value was 0.0190 µmole/mg of protein/min. In response to 100 mM urea, the enzyme activity was 0.0333 µmole and for the respective control, the activity was 0.0237 µmole/mg of protein/min. Whenever, the extract was treated with 200 mM urea, 0.0309 µmole urease activity was observed. On the contrary, flower extract without any urea treatment contained the urease activity 0.0237 µmol/mg of protein/min. As shown in Fig. 1 and

Table-2, urea causes an increase in enzyme activity by 31.57%, 40.50% and 30.37% for 50, 100 and 200 mM concentration when compared to the respective controls. The results demonstrated that 100 mM urea had enhanced the urease activity compared to other doses. Urease activity was also examined by using 400 µL flower extract where the effects of 50, 100 and 200 mM urea were done. The urease activity in response to 50 mM urea was 0.0178 µmole while 0.0244 µmole for 100 mM and 0.0196 µmole/mg of protein/min for 200 mM were found. On the contrary, flower extract without any urea treatment contained the urease activity 0.0130 µmol, 0.0166 µmol and 0.0142 µmol/mg of protein/min respectively. As shown in Fig. 2 and Table-3, urea causes an increase in enzyme activity by 36.92%, 46.98% and 38.02% for 50, 100 and 200 mM concentrations when compared to the respective controls. However, 100 mM urea predominantly stimulated the urease activity compared to other doses thereby the dose might be an optimum for the urease activity. The urease activity might be regulated by the variation of temperature and be strictly followed by the availability of urea in the soil. Therefore, it is reasonable that the growth of mango tree along with the production of flower and other necessary development might be improved because of the higher uptake of urea in the soil.

K_m and V_{max} of urease

The K_m and V_{max} were calculated by using the Lineweaver-Burke plot also called a double-reciprocal plot for the enzyme urease. If $1/V_o$ is plotted versus 1/[S], a straight line is obtained. The equation describing the Lineweaver-Burke plot is $1/V_o = K_m/ (V_{max} [S]) + 1/V_{max}$ where the intercept on the x axis is equal to $-1/K_m$ and the intercept on the y axis is equal to $1/V_{max}$. Determination of K_m and V_{max} is an essential parameter for characterization of enzyme activity. The K_m and V_{max} were determined by different doses of substrate (50, 100 and 200 mM urea) and enzyme extract. Table-4 and Fig. 3 show the K_m and V_{max} at 400 µL enzyme extract where the K_m value and the V_{max} were 62.5 mM and 0.04 µmol/mg of protein/min respectively. The results demonstrate that both K_m and V_{max} values are essential to determine the mechanism of action of enzyme urease in such circumstances.

Effect of urea on protein content in flower

Urea is widely used as N-fertilizer for production of agricultural crops because of its growth promoting effect. Therefore, it is generally recognized that urea may induce the synthesis of protein and other constituents. To examine whether urea is involved in inducing the synthesis of protein in flower,

the extract of flower (400 µL) was treated with urea and the amount of protein was determined following the method of Lowry *et al*. (1951). In response to 100 mM urea, the protein content was 15600 mg/100 g of flower while in the control without urea treatment, the amount of protein was 13200 mg/100 g of mango flower. The results (shown in Fig. 4) indicated that the amount of protein in flower sample had been increased by 18.18% which shows clearly that protein synthesis was stimulated after applying urea compared to the control.

Effect of urea on primary mono amine content in flower

Primary monoamine has been recognized to be a stimulatory factor for fertilization of mango flower and protective functions. To examine whether urea induces the synthesis of primary monoamine, extract of flower was used for the test of primary monoamine. Test of primary amine was performed in flower extract using diluted HCl and 10% $NaNO_2$. An orange reddish color was found demonstrating the presence of aromatic primary amine, however, in presence of 100 mM urea along with other necessary components, deep color was developed in the assay mixture and the color was more intensified than the control. The result indicates that the formation of primary amine is enhanced by the addition of urea. As shown in Fig. 5, the higher color development was found in the tube containing the flower extract with the dose of 100 mM urea compared to the control having no urea treatment.

Discussion

The present study reveals the mechanism of identification of enhancing fertilization of mango flower in response to different doses of urea. The higher activity of urease in presence of different doses of urea (50, 100 and 200 mM) was observed however, 100 mM urea was found to be involved in enhancing the enzyme activity greatly and therefore, the dose is optimum for higher activity of urease. The results also indicate that 100 mM urea induces the higher uptake of urea caused by the increased activity of urease. It is assumed that the uptake of nitrogenous compound in flower is mediated by administration of urea, there by inducing the synthesis of protein because NH_4^+ is directly correlated to the formation of protein in the cell. The disappearance of soil urea is the result of the higher formation of protein in the plants and causing the biomass of plants. The previous investigations reveal that foliar spraying of urea and KNO_3 significantly increased the flowering percentage of mango and generally KNO_3 has been shown to give better results in flowering and fruiting (Khattab *et al*., 2006). Jain (2006) indicated that single and double

spray treatment with 4% urea gave maximum yield in Madhya Pradesh, India. The plants treated with 4% urea solution exhibited the highest self life which was closely followed by KNO_3 at 4%. Irrespective of concentration, both urea and potassium nitrate manifested a marked in shelf life when compared with the plants under control.

Urea has been recognized to be a potent N fertilizer inducing the growth of plants as well as activators of many biological processes. The nitrogen availability and internal distribution plays a critical role in the regulation of various growth-related and morphogenetic aspects of plant development that are usually attributed to hormonal factors. Increased N supply stimulates plant growth and productivity, as well as photosynthetic capacity of leaves through increased amounts of stromal and thylakoid proteins in leaves (Hikosaka, 2004). Watcharasak and Thammasak (2005) found that fertilization with higher N supply gave the highest leaf number, leaf area, fresh and dry weight of shoot and roots in cucumber. An increase in nitrogen application resulted in maximum fruit length, fruit weight, vine length and yield of cucumber as demonstrated by Ahmed *et al.* (2007). The primary role of urease is to allow the organism to use external or internally generated urea as a nitrogen source. Significant amounts of plant nitrogen flow through urea. This compound derives from arginine and possibly from degradation of purines and ureides. The nitrogen present in urea is unavailable to the plant unless hydrolyzed by urease. The product of urease activity ammonia is incorporated into organic compounds mainly by glutamine synthetase. Urease catalyzes the conversion of urea into ammonia, which is subsequently assimilated by the plant via glutamine synthesis. The main function of plant ureases is thought to be related to nitrogen recycling from urea either formed endogenously or derived from external sources (Sirko and Brodzik, 2000; Follmer, 2008). Urease also has a fundamental role in recycling exogenous urea used as fertilizer (Witte *et al.*, 2002). The findings from the current investigation reveal that the flower extract is a good source of urease which uses urea as a substrate for its catalytic activity. The enhanced activity in response to urea may induce the growth of flower along with other necessary parameters regarding fruiting and fertilization process.

In analysis of mango flower, the different nutritional components were identified particularly the flower extracts had a higher amount of protein. Therefore, the mango flower is a good source of protein and of other nutritional requirements. The higher uptake of urea in flower might be an index causing higher protein synthesis, thereby increasing the enhanced growth. The results show clearly that 100 mM urea increased protein content in the flower whenever they were incubated with such dose of urea. Therefore, the results

correspond to the linearity between activation of urease and synthesis of protein and this linearity is affected by administration of urea. Although it is not clarified which type of protein is induced, however recent study reveals that urea promotes the higher expression of urea transporters in plants. To date, molecular identities of urea transporters in higher plants have been studied only in the dicot-model plant *Arabidopsis thaliana*. The transporter-mediated urea uptake in *Arabidopsis* roots with a high affinity and low affinity was recently affirmed by quantitative and short-time influx assays of [^{15}N]urea (Kojima *et al*., 2007). Molecularly, two types of urea transporting proteins have been reported in plant (Kojima *et al.*, 2006). It is assumed that urea uptake in flower might be mediated through the identified urea transporting system.

The nitrogenous compound primary amine is considered to be the agent causing fertilization in flower. Because of the deficiency of N-fertilizer, the synthesis of primary amine might be impaired; therefore the effect of urea on primary monoamine synthesis was performed in the study. Formation of primary amine in mango flower might be influenced not only by chemical factor but also by environmental stimuli. The flowers preferentially blossom during winter season and the onset of spring particularly at the end of January. Therefore, it is reasonably speculated that temperature might be involved in inducing the formation of primary amine. Environmental low temperature has been shown to be involved in the regulation of diverse metabolic functions in different organisms. It has been revealed that temperature variation is a common environmental phenomenon causing diverse metabolic alterations in plants and other organisms (Janska *et al*., 2010). Changes in environmental temperature affect the plant kingdom either by suppression of their total growth and development or by augmenting diverse physiological, metabolic and superficial changes. Low temperature has been recognized to be involved in regulation of thermogenesis while the thermogenesis is caused by the higher expression of UCP molecules (Holtzapffel *et al.*, 2002). The spadix in flower is the site of formation of heat during fertilization and the molecules responsible for this heat generation were considered to be the amine compounds (Dey and Harborne, 1997). Aromatic primary amine content was assumed to be enhanced in flower in our experiment when used with 100 mM urea along with other necessary compounds in assay mixture. Therefore, urea and other N-fertilizer may induce the synthesis and elicit the expression of UCP, a thermogenic protein causing thermogenesis. Further studies are needed to clarify the thermogenic mechanism during flowering of mango. It was found from the current investigation that 100 mM urea induced the synthesis of this compound and gave a deep yellow color compared to the control where no extracts were used and light yellow color was developed. As this compound is believed to be

involved in inducing the fertilization process, therefore urea application may induce the higher mango production. The following evidences may support the current investigation. Gupta and Brahmachari (2004) found maximum yield using urea. Moreover, fruit size, fruit weight and yield were effectively influenced with spraying of urea at Bihar, India signifying the role of urea in mango production. Potassium nitrate concentrations especially in combination with urea gave better results for most of the flowering and yield parameters of 'Tommy Atkins' mango in Ethiopia (Yeshitela *et al.,* 2005).

In *Arum maculatum*, flower morphology, primary and secondary metabolisms are highly co-ordinated to achieve a successful fertilization. The spadix is rich in starch. When the flower is mature, starch is broken down rapidly, resulting in pronounced thermogenesis. As a result, the club of spadix becomes quite warm. This is important for the dissipation of monoamines which are synthesized just at this time by action of a mitochondrial alanine-aldehyde amino transference. Primary monoamine has been recognized to be stimulatory factor for fertilization of mango flower and protective functions (Dey and Harborne, 1997). It is well known that even after havoc flowering in mango tree, percentage and numbers of mango fruits are very low, of course, adverse environmental factors are responsible for this effect. The flowers can not survive in that circumstances or lack of proper fertilization process is involved however, enhanced monoamine concentration in response to urea administration may augment the relief of these complications.

Conclusion

Although different doses of urea were used in this study, however, the results reveal that 100 mM concentration plays the critical role on enhancing urease activity in mango flower extract. Urea induced urease activity was found to be co-related to the synthesis of protein. Because, the amino nitrogen derived from urea during catalysis of urease is metabolized to synthesize protein in cells of plants. The increase activity of urease shows the higher uptake of urea in the flower because higher concentration of NH_4^+ is formed in response to urea. Primary monoamine has been believed to be involved in fertilization of mango flower and other protective functions. The higher color pigmentation was demonstrated in response to 100 mM urea treatment during the assay showing the higher synthesis of primary monoamine in flower induced by urea thereby causing fertilization and fruiting.

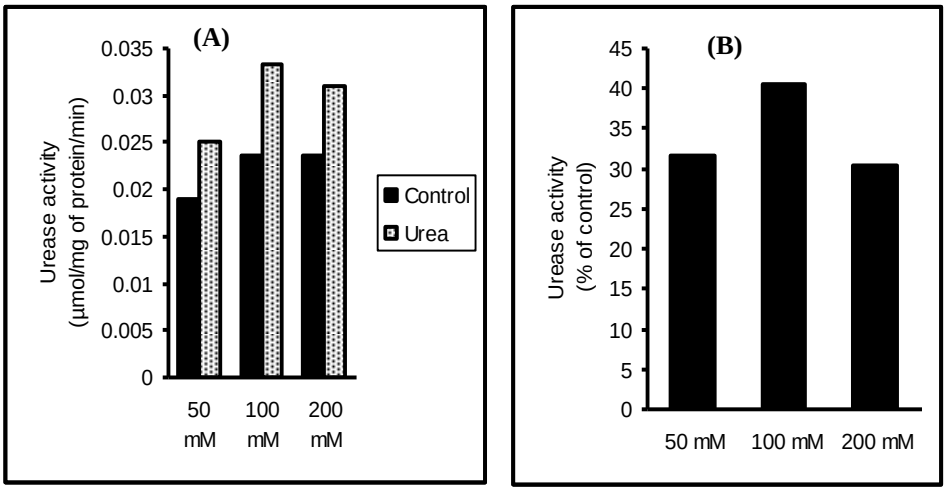

Fig. 1. Effect of different doses of urea in flower extract (200 µL) on urease activity (A). Percentage of increase in urease activity in flower in response to urea administration has been demonstrated (B).

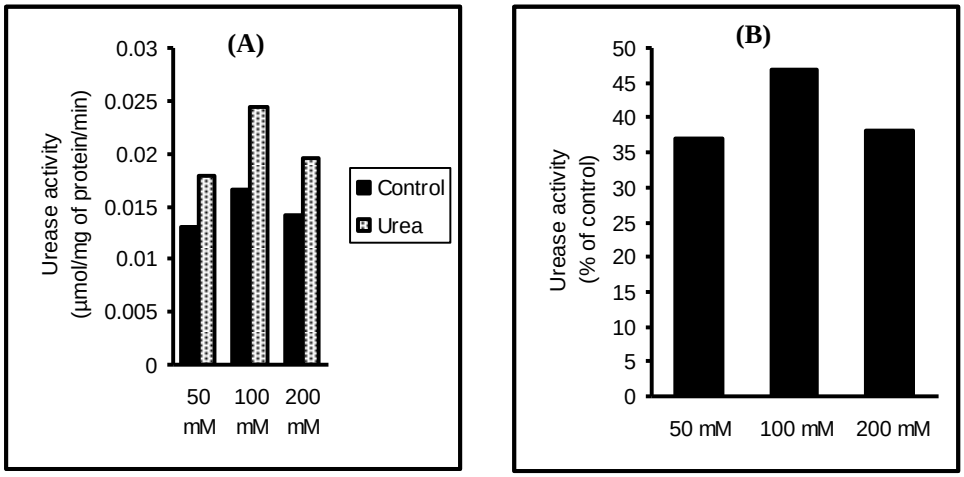

Fig. 2. Effect of different doses of urea in flower extract (400 µL) on urease activity (A). Percentage of increase in urease activity in flower in response to urea administration has been demonstrated (B).

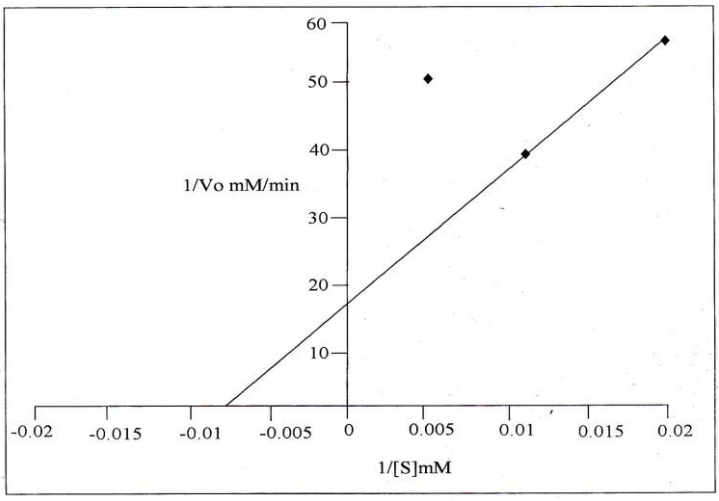

Fig. 3. Lineweaver-Burk double reciprocal plot for the determination of K_m and V_{max} of urease in 400 µL extract of mango flower. The equation describing the Lineweaver-Burke plot is $1/V_o = K_m/ (V_{max} [S]) + 1/V_{max}$ where the intercept on the x axis is equal to $-1/K_m$ and the intercept on the y axis is equal to $1/V_{max}$.

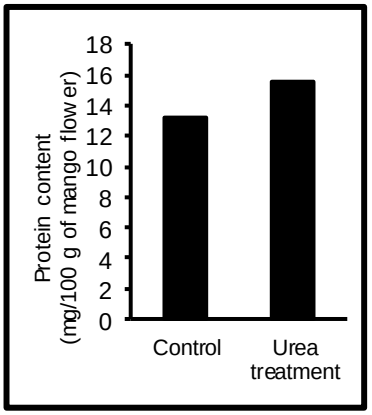

Fig. 4. Effects of urea on protein synthesis in extract of mango flower. 400 µL flower extract were treated with 100 mM urea and was incubated with 55 ^oC for 15 min. Control tube was similarly used except urea. After incubation, the above mixture was cooled and the protein content in 100 µL of the above sample and control was determined by the procedure of Lowry *et al.* (1951).

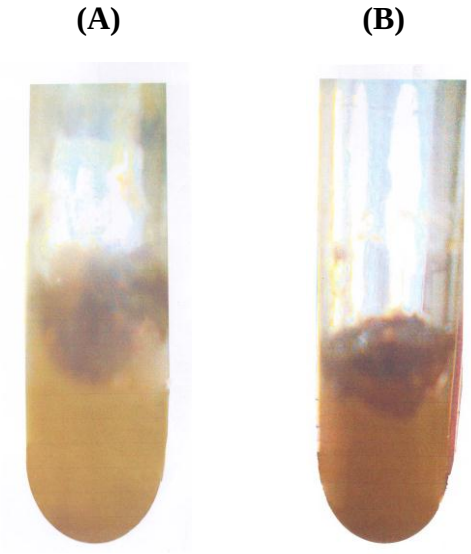

Fig. 5. Primary monoamine synthesis in mango flower for control (A) and induced by 100 mM urea (B).

Table 1. Nutritional analysis of mango flower

Parameter	**Weight of mango flower (g)**	**Total amount in flower**
Water soluble protein (g/100 g of mango flower)	5	12.0
Moisture (%)	2	77.15
Lipid (g/100 g of mango flower)	2	1.21
Total sugar (g/100 g of mango flower)	5	2.65
Reducing sugar (g/100 g of mango flower)	5	0.01
Non reducing sugar (g/100 g of mango flower)	5	2.64
Starch (g/100 g of mango flower)	5	4.01

Table 2. Urease activity in 200 µL mango flower extract in response to different doses of urea.

Substrate concentration (mM)	Absorbance at 680 nm		Enzyme activity (µmol/mg of protein/min	
	Control	Sample	Control	Sample
50	0.08	0.105	0.0190	0.0250
100	0.10	0.14	0.0237	0.0333
200	0.10	0.13	0.0237	0.0309

Table 3. Urease activity in 400 µL mango flower extract in response to different doses of urea.

Substrate concentration (mM)	Absorbance at 680 nm		Enzyme activity (µmol/mg of protein/min	
	Control	Sample	Control	Sample
50	0.12	0.15	0.0130	0.0178
100	0.14	0.205	0.0166	0.0244
200	0.12	0.165	0.0142	0.0196

Table 4. K_m and V_{max} of the enzyme urease in flower extract in response to different doses of urea

Substrate concentration (mM)	Extract volume (µL)	K_m (mM)	V_{max} (µmol/mg of protein/min)
50, 100 and 200	400	62.5	0.04

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