The interaction effect of Salt stress and Lysine Concentrations in some properties of Fenugreek callus

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Abstract. The callus induction by use 1.5mg/l of α-napthalene acetic acid (NAA) as auxin and 2mg/l of BA as cytokinins were added to MS medium that prepared as above before sterilize step. Sodium Chloride and Lysine effect on callus induction and growth of Fenugreek (Trigonella foenum-graecum L.).Five concentration of Sodium Chloride Salts (0, 2, 4, 6 and 8) were added to the culture medium and five concentrations of lysine (0, 10, 20, 30and 40mg/l) added to cultured medium. Results showed that NaCl at (2g/l) increased callus induction, fresh weight, dry weight and potassium ions of callus. The study also showed that (2 g/l) of NaCl was signification increased Sodium and Chloride ions of callus. Lysine (Lys) effect on callus characteristics:-The treatment 40mg/l outperformed of the rest treatments including control by gave the highest values of callus fresh and dry weights, as well as ions, soluble carbohydrates

Keywords. Trigonella foenum, callus, lysine, salinity, stress.

1. Introduction
Fenugreek plant (Trigonella foenum-graecum L.) of the family Fabaceae well known for their alkaloids they produce, some of these alkaloids exhibit interesting pharmacological and biological activities [1].
Fenugreek seeds have been extracted for polysaccharide, galactomannan, saponin, diosgenin, yamogenin, mucilage, volatile oil and alkaloids such as choline and trigonelline [2]. Sterol, diosgenin derivatives, furostanol glycosides which are responsible for its bitter taste of Fenugreek seeds, also folic acid, vitamin A, carotene, vitamin C, niacin, calcium potassium, phosphorus, iron, magnesium, and sodium as contains in seeds [3]. The plants in outer surrounding expose to various biological and non-biological stress, which showed adverse abnormal effects of plant by survival and the plant growth [4]. Physiological, morphological, microbial, physical and chemical factors which were induced or enhanced synthesis of secondary metabolites and growth improve called Elicitors, Elicitation is a process of Elicitors action. The application of elicitors, which is currently the focus of research, considered as one of the most effective methods to improve the synthesis of secondary metabolites in medicinal plants such of these elicitors are amino acids [5]. Salt stress can be define as old activator of secondary metabolites [6].

2. The role of amino acids in plant culture growth
Amino acids can play an important role in the development, establishment, precursors, elicitors and provide plant cells with a source of nitrogen easily assimilated by tissues and cells faster than inorganic nitrogen sources in culture system [7]. L-glutamine, L-asparagine, lysine and adenine are sources of organic nitrogen in culture media [9]. The Murashige and Skoog’s [MS] basal medium does not additional amino acid except glycine but in sometime added the coconut milk or casein hydrolysate which contain mixture of amino acids [8]. Amino acids have important role in plant life, such as enzymes molecules, plant cell division as well as stimulator, precursor of tannins, alkaloids, flavonoids, tocopherol and antienviromental stress, for example Phe is enter in 30% to supply and fixing carbon in the plant cell wall, lignin and cellulose forming [10].

Amino acids profiles in tissues, callus, suspension cells, and protoplasts culture of the plant species [11]. Amino acids used for enhancement of cell growth in culture media [9], callus culture and subcultured of phoraceae has been developed from leaf derived in amino acid [AA] basal medium with 1uM of 2,4-D [12]. The callus culture of Avicennia alba was induced from cotyledons in a modified amino acid [mAA] medium which difference MS in forms of nitrogen compounds and amino acids were added glycine, glutamine, aspartic acid, Lysine and arginine instead NH4NO3 and KNO3 in MS [13].

3. Amino Acids as a Precursor of Alkaloids
Amino acid can play an important role as a precursor of alkaloids and antienviromental stress [10]. Alkaloids may be classifying according to Amino acid precursor such as Phenylalanine precursor atropine, proline precursor ornithine, lysine precursor lysine derived alkaloids and tryptophan use as precursor Indole alkaloids group [14]. Tyrosine was use in Papaver somniferum callus to morphine alkaloid production and the best of conc. of Tyrosine was 30mg/l [28]. Hadi and others found that callus culture of medicinal plant catharanthus rosseous showed the ability of cells to produce the highest conc. of alkaloids compounds in accumulate media contain 0.5 mg/l tryptophan amino acids [15]. Lysine (Lys) (C6H14N2O2), is one of essential amino acids in protein structure, very freely soluble in water. (Molar mass 146.19 g/mol) colourless, crystals [16]. The oxaloacetate/aspartate family of amino acids asparagine, methionine, threonine, isoleucine and Aspartate can be converted into lysine [17]. Lysine decarboxylase catalyzes the first step of Quinolizidine alkaloid biosynthesis and alkaloid production in leguminosae [19]. Quinolizidine alkaloids (QAs) are usually known as lupine alkaloids synthesized by plants from lysine [18]. In belladonna callus culture, add Lys to medium was significantly increased fresh dry weight and absorption of calcium at last increase alkaloids production [20, 21, 22].

4. Materials and Methods
4.1. Source of Fenugreek plant Seeds
The fenugreek plant seeds obtained available in the commercial market. The seeds viability tested by germinated on filter paper witted with distilled water in a petri dish in the dark for three days in temperature 25±2°C.

4.2. Seed Surface Sterilization
Seeds were sterilized in 70% ethanol for 25 sec and 2% hypo chloride sodium solution for 10 minute seeds were then rinsed several times in sterile water. Increasing concentration of (2%) of NaOCl solution were used to sterilization of explant for 20 minutes, then washing with distilled water three times to remove the pernicious influence of sterile material. all operations were carried out in sterile conditions inside air flow class. The sterile explants were stitched into sterile MS culture media. 30 tube for each replication, then the cultures were incubated in darkness cooler incubator 25±.

4.3. Seed Germination and Explant
After seeds surface sterilization they were transferred to culture bottle and directly inoculated on the basic medium MS (Murashige and Skoog’s, 1962) salts and vitamins. Supplement with plant growth regulator, 1.5mg/l of α-naphthalene acetic acid (NAA) as auxin and 2.0mg/l of BA as cytokinins at 25 ±1°C incubate with dark conditions.

4.4. Callus Initiation. Explant Sterilization.

4.4.1. The MS Medium (Murashige and Skoog, 1962)
The Ready MS medium was used, the pH degree adjusted to 5.7+0.1 by using suitable standard sodium hydroxide (NaOH) or standard hydrochloric acid (HCl), then 7g of agar was added per 1 liter, then the MS was boiled by Magnetic stirrer hot plate. When the culture media become ready to used, 10ml of MS was added to each screw cup and covered and sterilized by steam sterilization (Autoclave) at 121°C for 20 minutes, then left the screw cup at room temperature, thus the culture media became ready for planting.

4.4.2. Sub-Culture of Callus in Accumulation Media
Accumulation media was prepared by adding 1.5mg/l of NAA and 2mg/l of BA to MS medium and different concentration of NaCl (0, 2, 4, 6, and 8) g/l. Each different concentrations of NaCl were mixed with (10, 20, 30, and 40) mg per liter of Lysine [23]. 150 mg of callus from stock cultures were used to prepared sub-culture in accumulation media, Incubated culture at growth conditions for 45 days.

4.4.3. The Proline mg/g Content
The matured callus of each treatment was estimated using a rapid colorimetric method as suggested by Bates et al., (1973). A fresh callus (0.5g) was homogenized in a mortar with 5ml of 3% sulfosalicylic acid. The homogenate was centrifuged at 10, 000 rpm for 10 min at 4C. The supernatant was diluted to 10ml with double-distilled water. Then 0.1ml of the diluted extract was placed in a test tube and further diluted to 1ml followed by addition of 5ml each of acid ninhydrin reagent and glacial acetic acid; the tube was boiled for 1h by a hot water bath. The reaction was terminated by keeping the test tube in an ice bath followed by addition of 4ml of toluene and stirred vigorously for 20-30S. The chromosphere-containing toluene layer (light pink) was aspirated from the aqueous phase and warmed to room temperature, and then the absorbance was read at 520nm on a UV-IS spectrophotometer by using pure toluene as a blank. The Proline concentration in the samples was determined from a standard curve prepared by using analytical grade Proline (Figure 9).

4.4.4. Total Carbohydrate Assay Kit
Glucose Standards for Colorimetric Detection Add 0, 2, 4, 6, 8, and 10 of the 2 mg/mL standard solution directly into a 96 well plate, generating 0 (blank), 4, 8, 12, 16, and 20 g/well standards. Add water to each well to bring the volume to 30. Sample Preparation Tissue (50 mg) or cells can be homogenized in
200 of ice-cold Assay Buffer. Centrifuge the samples at 13,000 for 5 minutes to remove insoluble material. Note: For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve. Bring samples to a final volume of 30 with water. Incubate the Assay reaction for 15 minutes at 90°C. Cover the plate and protect from light during the incubation. Then Add 30 of Developer to each well. Mix well using horizontal shaker for 5 minutes at room temperature. Mix contents for 1 minute before measuring the absorbance at A490 nm (Madhloom et al., 2018).

![Figure 1. A-D-Glucose Standard Curve. B- Standard Curve of Proline. C- Callus image](image)

5. Results

5.1. Callus Fresh Weight

Data in table (1) revealed that, the F.W. callus decreased with increasing NaCl. The highest value of F.W. callus was obtained from 2g/l NaCl treatment giving 2.81g, whereas the lowest value was obtained from 8g/l NaCl (1.19g). On the other hand, F.W. callus increased with increasing Lys from 0 to 40mg/l. While 0mg/l Lys treatment gave the lowest value of F.W. callus (1.94g), the 40mg/l Lys treatment gave the highest value of F.W. callus (2.85g). There was significant differs due to the interaction between the studied factors. F.W. callus was the lowest at 8g/l NaCl and 0 mg/l Lys giving 0.95g content. While F.W callus content at 2g/l NaCl and 40mg/l Lys was the highest giving 4.02g F.W callus.

| Lys. mg/l | NaCl concentrations g/l | Lys. Mean |
|----------|-------------------------|----------|
| 0        | 2.50 2.70 1.82 1.72 0.95 | 1.94     |
| 10       | 2.62 3.20 2.11 1.88 1.01   | 2.16     |
| 20       | 2.81 3.32 2.63 1.93 1.15   | 2.37     |
| 30       | 2.98 3.81 2.81 2.08 1.24   | 2.58     |
| 40       | 3.15 4.02 3.19 2.25 1.62   | 2.85     |
| NaCl mean| 2.81 3.41 2.51 1.97 1.19   |          |
| L.S.D. 0.05| NaCl = 0.634 Lys = 0.634 Interactin = 1.213 |          |
5.2. Callus Dry Weight

Data in table (2) revealed that, the D.W. callus decreased with increasing NaCl. The highest value of D.W. callus was obtained from 2g/l NaCl treatment giving 0.258 g, whereas the lowest value was obtained from 8g/l NaCl giving 0.171 g. On the other hand, D.W. callus increased with increasing Phe from 0 to 40mg/l. While o Phe treatment gave the lowest value of D.W. callus (i.e. 0.403g), the 40mg/l Phe treatment gave the highest value of D.W. callus (i.e. 0.117g). There was significant differs due to the interaction between the studied factors.

Table 2. The effect of NaCl and Lys on the D.W. Fenugreek callus

| Lys. mg/l | NaCl concentrations g/l | Lys. Mean |
|-----------|--------------------------|-----------|
|           | 0 | 2 | 4 | 6 | 8 |           |
| 0         | 0.129 | 0.150 | 0.115 | 0.100 | 0.091 | 0.117 |
| 10        | 0.183 | 0.214 | 0.193 | 0.160 | 0.151 | 0.180 |
| 20        | 0.236 | 0.250 | 0.240 | 0.199 | 0.181 | 0.219 |
| 30        | 0.274 | 0.328 | 0.402 | 0.260 | 0.211 | 0.275 |
| 40        | 0.315 | 0.369 | 0.320 | 0.291 | 0.220 | 0.403 |
| NaCl mean | 0.227 | 0.262 | 0.232 | 0.202 | 0.171 |           |
| L.S.D. 0.05 | | | | | | NaCl = 0.0143 Lys = 0.0143 Interaction = 0.0227 |

D.W. callus was the lowest at 8g/l NaCl and 0 mg/l Phe giving 0.091g content. While D.W callus content at 2g/l NaCl and 40mg/l Phe was the highest giving 0.369g D.W callus.

5.3. Proline in callus

Proline is a special case. Together with the α-C atom and the α-NH2 group, its side chain forms a five membered ring. Its nitrogen atom is only weakly basic and is not protonated at physiological pH

Table (3) showed that, the significantly effect of NaCl on Proline content, the highest Proline content at treatment (8 g/l NaCl) was (2.40 mg/g F.W.), whereas at treatment (0g/l of NaCl) which was recorded the lowest proline content ( 1.21 mg/g F.W.). The significant affected by Lys addition, the highest proline content at treatment of (0 mg/l Lys) reached (2.54mg/g F.W.), whereas the lowest value on proline content reached (1.10mg/g Lys F.W.) at treatment (40mg/l Lys).

Table 3. The effect of NaCl and Lys on proline content mg/g fresh weight in Fenugreek callus

| Lys. mg/l | NaCl concentrations g/l | Lys. Mean |
|-----------|--------------------------|-----------|
|           | 0 | 2 | 4 | 6 | 8 |           |
| 0         | 1.56 | 2.01 | 2.41 | 3.00 | 3.71 | 2.54 |
| 10        | 1.25 | 1.40 | 2.00 | 2.71 | 3.22 | 2.10 |
| 20        | 1.20 | 1.25 | 1.25 | 2.17 | 2.28 | 1.63 |
| 30        | 1.14 | 1.00 | 1.17 | 1.18 | 1.37 | 1.17 |
| 40        | 0.90 | 0.90 | 1.14 | 1.16 | 1.40 | 1.10 |
| NaCl mean | 1.21 | 1.29 | 1.59 | 2.04 | 2.40 | 2.54 |
| L.S.D. 0.05 | | | | | | NaCl = 0.122 Lys = 0.122 Interaction = 0.224 |

The interaction between NaCl and Lys, the highest value on proline content was (3.71 mg/g F.W.) at treatment (0 or 2 g/l NaCl and 40 mg/l Lys). While the lowest value on Proline content was (0.90 mg/g F.W.) at treatment (0 g/l NaCl and 10 mg/l Lys).

5.4. Total Soluble Carbohydrates

The results in table (4) showed significantly effect of NaCl on carbohydrates content, the highest value of carbohydrates content (16.026mg/g) D.W.at treatment (8g/l NaCl) while the lowest value (11.577mg/g) D.W.at treatment (0mg/l NaCl). Lys has significant increase on total soluble Carbohydrates values. The highest value reached (15.088mg/g D.W.) at 40mg/l Lys treatment. While
the lowest value of total soluble Carbohydrates values in 10mg/l was (14.503mg/g D.W.). The interaction between NaCl and Lys was significant, the highest value of Carbohydrates content was (16.400 mg/g D.W.), at treatment (4g/l NaCl) and (40mg/l Lys), whereas the treatment (0g/l NaCl) and (0mg/l Lys), gave the lowest value (10.162 mg/g D.W.).

Table 4. The effect of NaCl medium and Lys on content of total soluble Carbohydrate values mg/g D.W. Fenugreek callus

| Lys. mg/l | 0     | 2     | 4     | 6     | 8     | Lys. Mean |
|-----------|-------|-------|-------|-------|-------|-----------|
| 0         | 10.162| 15.242| 15.700| 15.980| 16.396| 14.696    |
| 10        | 11.122| 15.170| 15.721| 14.282| 16.218| 14.503    |
| 20        | 11.315| 15.630| 15.897| 14.35 | 16.021| 14.716    |
| 30        | 12.603| 15.851| 15.120| 14.522| 15.892| 14.798    |
| 40        | 12.682| 16.005| 16.400| 14.754| 15.601| 15.088    |
| NaCl mean | 11.577| 15.580| 15.768| 14.885| 16.026| 14.696    |
| L.S.D. 0.05|       |       |       |       |       |           |

5.5. Callus content of Trigonelline
Different levels of NaCl had significant effect on Trigonelline content (table 5), Where the control treatment gave the lowest value of Trigonelline content (0.154 mg/g D.W.), while the highest value of this active ingredient (0.305 mg/g D.W.) occurred at treatment 8g/l of NaCl. Lys addition to the growth medium has significant affected on Trigonelline content. The treatment 40mg/l Lys gave the lowest value of Trigonelline content (0.183 mg/g D.W.) while the highest value of this active ingredient reached (0.234 mg/g D.W.) occurred at treatment 10g/l of Lys. The interaction between these two factors was significant, where the control treatment gave the lowest value of Trigonelline content (0.111 mg/g D.W.) while the highest value of this active ingredient (0.396 mg/g D.W.) occurred at treatment 0mg/l of Lys and 8g/l of NaCl.

Table 5. The effect of NaCl medium and Lys on the content of Trigonelline in Fenugreek callus

| Lys. mg/l | 0     | 2     | 4     | 6     | 8     | Lys. Mean |
|-----------|-------|-------|-------|-------|-------|-----------|
| 0         | 0.111 | 0.214 | 0.220 | 0.230 | 0.396 | 0.234     |
| 10        | 0.218 | 0.210 | 0.217 | 0.233 | 0.350 | 0.246     |
| 20        | 0.223 | 0.225 | 0.209 | 0.219 | 0.300 | 0.235     |
| 30        | 0.128 | 0.220 | 0.196 | 0.211 | 0.250 | 0.201     |
| 40        | 0.090 | 0.202 | 0.182 | 0.202 | 0.237 | 0.183     |
| NaCl mean | 0.154 | 0.214 | 0.205 | 0.219 | 0.307 | 0.234     |
| L.S.D. 0.05|       |       |       |       |       |           |

6. DISCUSSION
After having been cultured in the medium for 3 days, callus began to form at the scutellum of the cultured seed. Most of the forming calli were compact type (Figure 1), they were milky in color and tightly aggregated showing their embryogenic calli nature.

The negatively effective may be due to the toxicity of ionic accumulation chloride and sodium [30]. In addition, the negative effect of salinity on callus weight may be attributed to lose of nitrogen content of callus, The salinity increase devastation by induce the analytical enzyme [33].The salinity has excessive production of the reactive oxygen species (ROS), which cause the oxidation of internal structures of cell, the cell metabolism that increase the speed of the respiration to produce additional
energy that necessary to adaptation with new osmosis conditions rather than use in the process of growth, as well as the more rapid respiration under the influence of stress affecting the flow of electrons and hence lead to the production of ROS (free radicals). The low molecular carbohydrates as soluble substances play important role in increasing of intracellular osmosis as a kind of tolerance to salt stress, this needs more energy that make callus limited its growth [6]. Similar results were reported by several researchers such as [42] on seedlings and calli of two Trigonella specie, [20] on potato [34]. Effect salt stress on carbohydrates accumulation, these organic solutes even at high concentration are not harmful to enzymes and cellular structures for this reason, these organic compounds referred to as compatible osmolytes, adjustment and balance outside, cytosol and vacuole of cell, compatible osmolytes may protect plants by scavenging oxygen free radicals [35].

Salt stress effect on proline and trigonelline content as defense way to help plant under stress from toxic effect of ammonia that synthesized during protein catabolism, consumption of ammonia to build high impact reduces proline in plant [36]. The increasing of proline due to an imbalance of cell osmosis that lead to product (proline) to equilibrium between the vacuole and cytoplasm, Proline acts as a protective enzymes and antioxidant, scavenges of free radicals, Proline may be in normal concentration despite of the plant in salinity medium, because enhanced growth under saline conditions in cultured such as barley embryos [38]. Increased proline under NaCl stress also recorded in comparison to control cultures of Salvadora persica [35]. Salt stress increases various secondary metabolites in plants as osmolytic like alkaloids [37]. Callus under NaCl conditions stimulates its cells to produce secondary metabolites and in larger quantities than the mother plant. Because of its physiological behavior [31]. Callus induction to alkaloids production under NaCl stress Alkaloids (trigonelline) content productions are act osmolytes and osmoprotectants [35]. And the alkaloids considers as a nitrogen source. Similar results were reported by several researchers such as, the total alkaloid content was increase in Datura subjected to salt stress thus may be as alkaloid (trigonelline) that is increasing too [39]. Atropa belladonna in vitro, 40 on Hibiscus acetosella, Salvadora persica [41].

All MS media supplemented with different concentrations of Lysine gave high percentage of callus formation in the range of 91–95 %. L-lysine seemed to be the best to support callus formation, This leads to increased osmotic pressure, which leads to draw water from the neighboring cells and fill, the cell wall becomes soft and thin [31] there was no statistical difference among the results in using different amount of L-lysine in the cultured medium or even from L-lysine free medium. The significant role of L-lysine on callus formation, the catalytic groups are usually the amino acid side chains and/or cofactors that can function as catalysts, bases (—NH2 from lysine, the basic ones by accepting a proton. the side chains of the basic amino acids lysine are also fully ionized—i. e., positively charged—at neutral pH. therefore extremely polar. The basic amino acid ornithine is an analogue of lysine with a shortened side chain lysine residue is modified by hydroxylation to prepare for the formation of stable structures, as lipoamide marketed as antioxidant. Transfer of a carbamoyl residue to ornithine and lysine yields citrulline, which is intermediate in the urea cycle, with their high proportions of lysine Many coenzymes and cofactors are covalently linked to lysine residues, Its acetylation (or deacetylation) is an important mechanism for controlling genetic activity and histone protein stability, [32] Lysine—tRNA ligase catalyzes the specific attachment of an amino acid to its cognate tRNA in a 2 step reaction: the amino acid (AA) is first activated by ATP to form AA-AMP and then transferred to the acceptor end of the tRNA. [24]. Rubisco Activity Increases with an uncharged ε-NH2 group of lysine within the active site of the enzyme. Then rapidly binds Mg2+ to yield the activated complex [30]. Alkaloids are usually synthesized from one of a few common amino acids—in particular, lysine, tyrosine, and tryptophan [30]. Lysine stimulate that alkaloids enzymes synthesize [31]. The conjugated IAA to the amino acid lysine more active [29].

7. Conclusions
Study can be concluded Callus growth Stimulated at 2 g/l NaCl, and at treatment 4, 6, 8g/l NaCl inhibited callus growth. Callus growth stimulated at 40mg/l of Lys. trigonelline increased at treatments 40mg/l of Lys on other hand the highest value of trigonelline at 6 and 8 g/l NaCl .The difference in salt tolerances
between the studied stimuli may related to the control of genetic factors that would influence the characteristics of the callus growth, as well as the ability of plant to accumulate of soluble carbohydrates, proline, ion content as well as trigonelline.

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