Synthesis of organic iron chelates and evaluation of their efficiency in improving the growth and yield of Blackgram

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Abstract
Supplying a sufficient amount of available iron (Fe) for plant growth in pot culture is a great challenge. The chelators commonly used to supply Fe in pot culture have several advantages and positively affect plant growth. In this research study we have synthesized certain Fe – amino acid and Fe – organic acid chelates, including Fe – glycinate, Fe – citrate and Fe – EDTA and evaluated their efficacy as a Fe source for blackgram grown in pot culture. FTIR analysis shows unbound glycine from chelated one. Free glycine exhibit a vibration peak at 2920 cm$^{-1}$ that disappear upon chelation formation. The peak at 2920 cm$^{-1}$ due to twisting vibrating of NH$_2$ groups. Disappearing of this peak indicates that new coordinate bond is formed through the terminal amine groups. The peak at 3153.04 cm$^{-1}$ and 1327.75 cm$^{-1}$ is due to weak stretching of symmetric and asymmetric amine vibration. As compared to the position of bands in the spectrum of iron glycinate confirms the chelation of said amino acid with Fe$^{2+}$ ions. The pot experiment was conducted to study the effect of foliar and soil application of amino acid and organic acid chelated iron on growth of blackgram. The results showed that the foliar spraying of 1% ferrous glycinate resulted in maximum Fe, Zn content, leaf active iron, Catalase and Peroxidase activity. Higher activity of Catalase and Peroxidase confirmed the improvement in plant Fe status. Between the soil and foliar application foliar spraying is better in improving the plant micronutrient content which was evidenced from higher enzyme activities.

Keywords: Ferrous glycinate, leaf active iron, FTIR, catalase, peroxidase, micronutrients

Introduction
Iron is one of the most important micronutrient necessary to life and growth of plants because it helps in the formation chlorophyll (Curie et al., 2008) [13] and enzymes (Rivero et al., 2005) [14]. Iron is a constituent of many enzymes like, cytochrome oxidase, catalase, peroxidase, acotinase, and nitrogenase. The deficiency of iron causes chlorosis leaves and exhibit in the young leaves of plants. Susceptibility to iron deficiency is varied with varieties of same crops. Crops such as sorghum, field beans and soybeans can show severe iron chlorosis, where corn or alfalfa may appear normal. Some new varieties of soyabean can use iron efficiently than other, therefore selecting the appropriate crop and variety is important (Schulte and Kelling, 2004) [17]. Decomposition of organic matter releases some chelating agents like organic acids, amino acids, ligininsulfonates, ligninpolycarboxylates, sugar acids, derivatives, phenols, poly flavonoids, siderophores and phyto siderophores. Both classes of chelating/complexing agents increase micronutrient solubility (Sekhon, 2003) [18]. Chelating agents like citric acid and gluconic acid have the capacity to form water soluble chelates due to the presence of carboxylic and hydroxyl group combined with metal ions over coordinated covalent bonding. The hydroxyl groups are unattached complex with the metal ions under slightly acidic conditions (Clemens et al., 1990) [4]. Calcium salts in soils have been found to increase the sorption of chelates (Wallace and Lunt, 1956) [21]. With increased concentrations of Ca-salts, there is a suppression of electrical double layer around the negatively charged surfaces, which permit anionic species to adsorb to sorption sites (Bolt and Warkentin, 1958). Carboxylate is one of the constituent in the root exudates which act as a chelating agent to alleviate nutrient deficiencies (Marschner and Römheld, 1994) [19].
Results from foliar applications may be only temporary and actually depress the plant’s Fe stress mechanisms by preventing the increase in Fe-reducing capacity of the roots that would normally occur during Fe deficiency (Römheld and Marschner, 1986) [13]. In solutions where Ca is the only competing cation, HEDTA was found to be a more effective chelate than EDTA. Godsey et al. (2003) found that foliar-applied chelated Fe-HEDTA was not effective in increasing grain yield, indicating that another Fe source should be considered. However, seed-applied chelated Fe fertilizer (HEDTA) increased grain yield by approximately 55 percent for both tolerant and non-tolerant soybean varieties (Liesch et al., 2011) [9].

Amino acids are chelated with micronutrients responsible extreme bioavailability, acceptability and protection. These amino acid chelated micronutrients are exactly used for foliar spraying and soil application. Schaffer et al. (2011) [16] examined that the natural amino acids are very small molecules they chelated with micronutrients therefore they are quickly absorbed, translocated and metabolized by plants. The merits of amino acid chelates are that the amino acid ligand frame and defend the micronutrients from adverse relations usually those take place in soil solution, in the presence of soil or on leaf surfaces. Sekhon (2003) [18] reported that the amino acid chelates are neutral charge and surface of the leaves negatively charged. They are neither attracted to nor repulsed from leaf surface and liberally permit through this barrier. So, amino acid chelate is taken into the cell very quickly and efficiently. Keeping this in view the present study was carried out to prepare amino acid Fe chelates and evaluated their efficacy on blackgram growth and yield.

Material and Methods
Synthesis of iron chelates
1,000 grams of water was boiled for 30 minutes to remove dissolved air and 170 grams of ferrous sulfate monohydrate was dissolved in 500 ml of the de aerated water and the solution was maintained at 80°C and 30 grams of citric acid was mixed to it. Separately 150 grams of glycine was dissolved in 500 ml of de aerated water and the acid solution was added to the ferrous sulfate solution with stirring. The temperature of the mixture was maintained at 80°C and the mixture was filtered to remove any undissolved materials. The metal amino acid citrate was dried at 110°C and the dry material was ground to a fine powder (Hsu, 1996) [17].

Chelate analysis
The analysis was carried out in FT-IR – 6800 (Jasco, Japan) equipped with ATR PRO ONE accessory and TGS detector. Registration was carried out in the region of 400 – 4000 cm⁻¹ (resolution 4 cm⁻¹ with 40 number of scans). The report was then processed using origin ® 8.0 software and interpreted.

Pot experiment
The pot experiment was conducted at Tamil Nadu Agricultural University, Coimbatore to find out the effect of amino acid and organic acid chelated iron on growth and productivity of blackgram. The seeds of blackgram were obtained from Department of Pulses, TNAU, Coimbatore and soils were collected from farmer’s field of Thondamuthur, Coimbatore. Seeds were sown in the pots at three seeds pots⁻¹ with nine treatments involving T₁ - NPK control, T₂ - FeSO₄ 25 kg ha⁻¹ as basal soil application, T₃ - Ferrous glycinate chelate @ 5 kg ha⁻¹, T₄ - Ferrous citrate chelate @ 5 kg ha⁻¹, T₅ - Fe – EDTA chelate @ 5 kg ha⁻¹, T₆ - 1% FeSO₄ as foliar spraying on 25 & 45 DAS, T₇ - 1% Ferrous glycinate as foliar spray on 25 & 45 DAS, T₈ - 1% Ferrous citrate as foliar spraying on 25 and 45 DAS, and T₉ – 1% Fe – EDTA as foliar spray on 25 and 45 DAS. Five randomly selected plants from net plot area were used to record the dry matter production at different growth stages. The sampled plants were partitioned into leaves, stem and pods and were dried at 65°C till they attained constant dry weight. Dry weight of stem, leaves, pods and total dry matter production per plant were recorded at all the growth crop stages and expressed as g per plant.

Elemental analysis
Plant samples were collected at three stages viz., vegetative, flowering and harvest stages, shade dried and then kept in a hot air oven at 65°C and ground in a Willey Mill. The processed plant samples were analyzed for total nutrient content of Fe, Cu, Mn and Zn by adopting standard procedure (Hessey, 1971) [10]. Nutrient uptake computed by multiplying the nutrient content of the plant with dry matter production and expressed on oven dry weight basis.

Active iron content
Two grams of chopped plant sample was weighed and transferred to 100ml glass bottles. Twenty ml of o-phenanthroline solution was added and the content of the bottles were stirred gently in order to embathe the plant sample with the extractant. The bottles were stoppered and allowed to stand for 16 hrs at room temperature. The contents were filtered through Whatman No. 1 filter paper. The active iron was estimated directly in the filtrate by measuring the transmittance at 510 nm in spectrophotometer (Katyal and Sharma, 1980) [8].

Estimation of catalase activity
Catalase activity was determined by following titration method using potassium permanganate (NC, 1963) and expressed as μg H₂O₂ g⁻¹ min⁻¹. 250 mg of leaf sample was macerated with 10 ml of sodium phosphate buffer (0.2 M, pH 6.8) and the content was centrifuged at 10,000 rpm at 4°C for 20 minutes. The supernatant solution of 1 ml was taken in four beakers, 5 ml of 1.5 per cent sodium perborate, 1.5 ml of sodium phosphate buffer (0.2 M, pH 6.8) and 10 ml of sulphuric acid was added in a first four beakers at the time interval of 1, 2, 3, 4 minutes respectively. In 5th beaker, 10 ml of sulphuric acid was added first, then 5 ml of sodium perborate (1.5%), 1.5 ml of sodium phosphate buffer (0.2 M, pH 6.8) and 1 ml of enzyme extract was taken and kept as blank. The solution was titrated against with 0.05 per cent potassium permanganate and the development of pink colour, which persists for 30 seconds, is the end point.

Estimation of peroxidase activity
Peroxidase activity (change in absorbance value at 430 nm g⁻¹ min⁻¹) was determined according to Purur (1962) [13] and Angelini et al. (1990) [11]. 100 mg of leaf sample was extracted using 0.1M phosphate buffer (pH 7.0) and a known volume of the extract was added to a cuvette containing 3 ml phosphate buffer and 3 ml pyrogallol and increase in absorbance at 430 nm was recorded. The change in absorbance in minutes was used to calculate the enzyme activity.

Statistical analysis
The data obtained from different experiments was analysed statistically to find out the effects of various treatments and...
their interactions. Analysis of variance was calculated as suggested by Panse and Sukhatme [1967] [12]. Simple correlation and regression co-efficient were also worked out between certain inter-related parameters to observe their degree of dependence as suggested by Snedecor and Cochran [1967] [19].

Results
Characteristics of iron chelates
The iron amino acid and iron organic acid chelates were synthesized by reacting of FeSO₄ and glycine, citric acid and EDTA in 1:2 mole ratio and the reaction yield was more than 80%. The amino acid ligand generally act as a bidentate (N, O) chelates with respect to pH. The FTIR spectra of the complexes show an absorption pattern in 4000 – 400 cm⁻¹ region, similar to amino acid ligands. FTIR analysis shows unbound glycine from chelated one. Free glycine exhibit a vibration peak at 2920 cm⁻¹ that disappear upon chelate formation (Fig 1).

Dry matter weight
The root and shoot dry matter production of black gram was influenced by the foliar spraying of 1% ferrous glycinate on 25 and 45 DAS and registered the highest mean root DMP of 132, 145, and 157 kg ha⁻¹ at vegetative, flowering and harvest stages respectively and highest mean shoot DMP (T₇) of 351, 385 and 415 kg ha⁻¹ at vegetative, flowering and harvest stages respectively, which was on par with soil application of ferrous glycinate at 5kg ha⁻¹ (T₃) (Table 1).

Leaf active iron content
The foliar spraying of 1% ferrous glycinate twice at 25 and 45 DAS at vegetative, flowering and harvest stages increase the leaf active iron content followed by soil application of ferrous glycinate at 5 kg ha⁻¹ (T₃). This influence is due to the application of higher concentration of iron at different stages and thereby increased the absorption of iron in black gram.

Iron content
The gradual increase in iron content of above ground bio mass with advancement of growth has observed. The foliar spraying of 1% ferrous glycinate on 25 and 45 DAS at vegetative, flowering and harvest stages increase the shoot and root iron content (Fig 2). Increased DMP coupled with higher iron concentration resulted in higher uptake of Fe in the present investigation. Foliar spraying of 1% ferrous glycinate @ 25 & 45 DAS recorded the maximum iron uptake by both shoot and root at all the growth stages.

Zinc content
The different iron chelates brought out significant variations in the content of zinc. The highest mean shoot zinc content of 14.4, 17.3 and 16.1 mg kg⁻¹ at vegetative, flowering and harvest stages respectively was recorded with 1% of ferrous glycinate as foliar spraying on 25 and 45 DAS (T₃). The highest mean root zinc content of 7.40, 10.5 and 9.70 mg kg⁻¹ at vegetative, flowering and harvest stages respectively was recorded in the treatments that received 1% ferrous glycinate as foliar spraying on 25 and 45 DAS (T₃) (Fig 3).

Enzyme activity in plants
Foliar spraying of 1% ferrous glycinate registered the highest mean catalase activity of 11.4 and 14.8 µg of H₂O₂ g⁻¹ min⁻¹ at vegetative and flowering stages (Table 2) and on par with soil application ferrous glycinate at 5 kg ha⁻¹. The enzyme peroxidase activity showed an upheaval trend for the application of iron chelates. Foliar spraying of 1% ferrous glycinate (T₃) registered the highest mean peroxidase activity of 6.90 and 7.40 ∆ 430 nm min⁻¹ g⁻¹ at vegetative and flowering stages (Table 2).

Discussion
The peak at 2920 cm⁻¹ due to twisting vibrating of NH₂ groups. Disappearing of this peak indicates that new coordinate bond is formed through the terminal amine groups. The peak at 3153.04 cm⁻¹ and 1327.75 cm⁻¹ is due to weak stretching of symmetric and asymmetric amine vibration. As compared to the position of bands in the spectrum of iron glycinate confirms the chelation of said amino acid with Fe²⁺ ions [Yin et al., 2017]. The iron amino acid and organic acid chelates were synthesized using Glycine, Citric acid, and EDTA. These are abundant in the plant rhizosphere and play significant role in plant and human nutrition of Fe. The stability of metal complexes of these amino acids is also high in water.

The root and shoot dry matter production of black gram was influenced by the foliar spraying of 1% ferrous glycinate on 25 and 45 DAS. The better performance of foliar spraying of 1% ferrous glycinate (T₃) at 25 and 45 DAS treatment on DMP in the present study could be due to the fact that the plants absorb and transport iron efficiently in its glycinate form i.e., glycine helps maintain the iron in its soluble form within the plants (Chavan and Banerjee, 1980) [3]. The foliar spraying of 1% ferrous glycinate twice at 25 and 45 DAS at vegetative, flowering and harvest stages increase the leaf active iron content. Similar results were reported by Shuedzhen [1991] [19] where the higher active iron content in the foliar application of 1% ferrous glycinate treatment could be due to the fact that plant absorbs and translocates iron efficiently in citrate form. Increased DMP coupled with higher iron concentration resulted in higher uptake of Fe in the present investigation. Foliar spraying of 1% ferrous glycinate @ 25 & 45 DAS recorded the maximum iron uptake by both shoot and root at all the growth stages which is in accordance with findings Yadav and Sehgal [2002] [22]. The supply of iron with higher concentration at critical stages resulting in increased absorption and translocation of zinc. In case of root the zinc content was found to decrease from flowering to harvesting stages, which can be attributed to dilution effect (Chavan and Banerjee, 1980) [3]. Higher activity in the presence of ferrous glycinate is due to the role of glycine expression of genes encoding the catalase activity. The activity of these enzyme increased with decreasing iron rates. Iron is a constituent of enzyme system and so it helps for carrying out different enzymatic reactions in plant like, cytochrome oxidase, catalase, peroxidase, acotinase, and nitrogenase. This has been proved by Zahra Karimi [2014] [23] who ascribed that comparison effect of nano-iron chelate and iron chelate on growth parameters and antioxidant enzymes activity of green gram.
Table 1: Effect of iron chelates on dry matter production at different stages of black gram (kg ha\(^{-1}\))

| Treatments                                      | Vegetative | Flowering | Harvest | % increase over control | Vegetative | Flowering | Harvest | % increase over control |
|-------------------------------------------------|------------|-----------|---------|-------------------------|------------|-----------|---------|-------------------------|
| T\(_1\) - NPK control                           | 320        | 359       | 391     | -                       | 105        | 119       | 135     | -                       |
| T\(_2\) - FeSO\(_4\) @ 25 kg ha\(^{-1}\) as basal soil application | 325        | 365       | 397     | 1.59                    | 115        | 125       | 145     | 7.24                    |
| T\(_3\) - Ferrous glycinate chelate @ 5 kg ha\(^{-1}\) | 346        | 376       | 409     | 5.70                    | 126        | 138       | 155     | 16.7                    |
| T\(_4\) - Fe – EDTA chelate @ 5 kg ha\(^{-1}\)   | 336        | 369       | 395     | 2.80                    | 117        | 128       | 141     | 7.52                    |
| T\(_5\) - 1% FeSO\(_4\) as foliar spraying on 25 & 45 DAS | 339        | 365       | 401     | 3.27                    | 119        | 127       | 139     | 7.24                    |
| T\(_6\) - 1% Ferrous glycinate as foliar spraying on 25 & 45 DAS | 329        | 362       | 403     | 2.24                    | 110        | 119       | 146     | 4.46                    |
| T\(_7\) - 1% Ferrous citrate as foliar spraying on 25 & 45 DAS | 351        | 385       | 415     | 7.57                    | 132        | 145       | 157     | 20.9                    |
| T\(_8\) - 1% Fe – EDTA as foliar spraying on 25 & 45 DAS | 340        | 373       | 405     | 4.49                    | 124        | 135       | 151     | 14.2                    |
| S.Ed                                            | 8.42       | 8.06      | 9.13    | 2.65                    | 3.75       | 2.35      |          |                          |
| CD (P=0.05)                                      | 17.6       | 16.9      | 19.1    | 5.57                    | 7.88       | 4.94      |          |                          |

Table 2: Effect of iron chelates on enzymes activity at different stages of black gram (µg of H\(_2\)O\(_2\)/g/min)

| Treatments                                      | Catalase activity (µg of H\(_2\)O\(_2\)/g/min) | Peroxidase activity (µA 430 nm min\(^{-1}\)g\(^{-1}\)) | Vegetative | Flowering | Vegetative | Flowering | Vegetative | Flowering | Vegetative | Flowering | Vegetative | Flowering | Vegetative | Flowering | Vegetative | Flowering | Vegetative | Flowering |
|-------------------------------------------------|-----------------------------------------------|--------------------------------------------------------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|
| T\(_1\) - NPK control                           | 3.83                                          | 6.80                                                   | 5.44       | 5.92      | 5.44       | 5.92      | 5.44       | 5.92      | 5.44       | 5.92      | 5.44       | 5.92      | 5.44       | 5.92      | 5.44       | 5.92      | 5.44       | 5.92      |
| T\(_2\) - FeSO\(_4\) 25 @ kg ha\(^{-1}\) as basal soil application | 10.2                                          | 12.7                                                   | 5.94       | 6.44      | 5.94       | 6.44      | 5.94       | 6.44      | 5.94       | 6.44      | 5.94       | 6.44      | 5.94       | 6.44      | 5.94       | 6.44      | 5.94       | 6.44      |
| T\(_3\) - Ferrous glycinate chelate @ 5 kg ha\(^{-1}\) | 10.6                                          | 13.1                                                   | 6.54       | 7.12      | 6.54       | 7.12      | 6.54       | 7.12      | 6.54       | 7.12      | 6.54       | 7.12      | 6.54       | 7.12      | 6.54       | 7.12      | 6.54       | 7.12      |
| T\(_4\) - Ferrous citrate chelate @ 5 kg ha\(^{-1}\) | 5.10                                          | 7.65                                                   | 6.10       | 6.68      | 6.10       | 6.68      | 6.10       | 6.68      | 6.10       | 6.68      | 6.10       | 6.68      | 6.10       | 6.68      | 6.10       | 6.68      | 6.10       | 6.68      |
| T\(_5\) - Fe – EDTA chelate @ 5 kg ha\(^{-1}\)   | 4.68                                          | 7.65                                                   | 5.51       | 6.00      | 5.51       | 6.00      | 5.51       | 6.00      | 5.51       | 6.00      | 5.51       | 6.00      | 5.51       | 6.00      | 5.51       | 6.00      | 5.51       | 6.00      |
| T\(_6\) - 1% FeSO\(_4\) as foliar spraying on 25 & 45 DAS | 8.50                                          | 11.4                                                   | 6.54       | 7.08      | 6.54       | 7.08      | 6.54       | 7.08      | 6.54       | 7.08      | 6.54       | 7.08      | 6.54       | 7.08      | 6.54       | 7.08      | 6.54       | 7.08      |
| T\(_7\) - 1% Ferrous glycinate as foliar spraying on 25 & 45 DAS | 11.4                                          | 14.8                                                   | 6.90       | 7.40      | 6.90       | 7.40      | 6.90       | 7.40      | 6.90       | 7.40      | 6.90       | 7.40      | 6.90       | 7.40      | 6.90       | 7.40      | 6.90       | 7.40      |
| T\(_8\) - 1% Ferrous citrate as foliar spraying on 25 & 45 DAS | 5.53                                          | 7.65                                                   | 6.74       | 7.16      | 6.74       | 7.16      | 6.74       | 7.16      | 6.74       | 7.16      | 6.74       | 7.16      | 6.74       | 7.16      | 6.74       | 7.16      | 6.74       | 7.16      |
| T\(_9\) – 1% Fe – EDTA as foliar spraying on 25 & 45 DAS | 8.08                                          | 11.9                                                   | 5.97       | 6.44      | 5.97       | 6.44      | 5.97       | 6.44      | 5.97       | 6.44      | 5.97       | 6.44      | 5.97       | 6.44      | 5.97       | 6.44      | 5.97       | 6.44      |
| Mean                                            | 7.54                                          | 10.4                                                   | 6.69       | 6.19      | 6.69       | 6.19      | 6.69       | 6.19      | 6.69       | 6.19      | 6.69       | 6.19      | 6.69       | 6.19      | 6.69       | 6.19      | 6.69       | 6.19      |
| SEd                                             | 0.02                                          | 0.05                                                   | 0.07       | 0.15      | 0.07       | 0.15      | 0.07       | 0.15      | 0.07       | 0.15      | 0.07       | 0.15      | 0.07       | 0.15      | 0.07       | 0.15      | 0.07       | 0.15      |
| CD (P=0.05)                                      | 0.06                                          | 0.11                                                   | 0.16       | 0.31      | 0.16       | 0.31      | 0.16       | 0.31      | 0.16       | 0.31      | 0.16       | 0.31      | 0.16       | 0.31      | 0.16       | 0.31      | 0.16       | 0.31      |

Fig 1: FTIR spectrum of Fe – Glycinate
Fig 2: Effect of iron chelates on iron content at different stages of black gram (mg kg\(^{-1}\))

Fig 3: Effect of iron chelates on zinc content at different stages of black gram (mg kg\(^{-1}\))

Conclusion
Foliar application of 1% ferrous glycinate increased the dry matter production, leaf active iron, enzyme activity and micronutrient content of iron, zinc. The use of organic iron chelates for the correction of micronutrient deficiencies has increased in farms, as chelated micronutrients are soluble in solution and do not enter into adverse reaction in soil and easily absorbed and translocated by plants. Amino acid chelated micronutrient deliver selected nutrients with maximum bioavailability and safety to environment.

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