J Agric. Res., 2018, Vol. 56(1):27-34
www.jar.com.pk
Agriculture Department
Government of Punjab

SOIL SCIENCE

BIOREMEDIATION OF HEAVY METAL STRESS BY *RHIZOBIUM* CHICKPEA SYMBIOSIS

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Article received on: 21/02/2016
Accepted for publication: 23/06/2017

ABSTRACT

A pot experiment was conducted in Soil Bacterology Section, Ayub Agricultural Research Institute, Faisalabad, Pakistan to study the role of *Rhizobium* in heavy metal remediation in two consecutive years (2013-14 and 2014-15). Growth and yield of chickpea, under metal contaminated soil, was evaluated. At the time of pot filling copper was applied as $CuSo_4.5H_2O$ @ 50, 100,150 and 200 mg kg^{-1} soil in all the treatment. In these treatments, chickpea seed was inoculated with peat based *Rhizobium* inoculums. Fertilizers @ 30-60 kg NP ha^{-1} were applied at sowing. The trial was laid out according to CRD having four repeats. The results revealed that post-harvest soil carried lower Cu contents compared to pre-sowing soil even without inoculation. However, *Rhizobium* inoculation decreased the Cu contents upto 73-98%. Significant increase in grain yield (16.8 g pot^{-1}) was observed by *Rhizobium* inoculation at Cu concentration of 50 mg kg^{-1}. The physical parameters of the plants like root/shoot length, biomass, dry weight, nodular mass and number of nodules $plant^{-1}$ were also increased significantly at all Cu levels combined with *Rhizobium* inoculation. It is concluded that rhizobial inoculation exerted positive effect on growth of crop in metal infected soil.

KEYWORDS: *Cicer arietinum;* chickpea; heavy metal; Cu; bioremediation; *rhizobium;* yield; Pakistan.

INTRODUCTION

Naturally occurring elements have negative, positive or no effect on the crop growth. Some of these elements are essential for life and rests are unexplored for biological functions. Metabolic metals are structural part of many proteins and enzymes, which play a significant role in physiology of the plants (Gambling *et al.,* 2004; Mehta *et al.,*2006). Some metals like Cu are toxic to microbes, plants and animals, even though, their presence is unavoidable for their growth. The non-metabolic metals obstruct the functions of physiologically required metals (Achard *et al.,* 2007). Metals have three key mechanisms by which these cause toxicity in biological systems i.e. dislodgment of metals biologically efficient metals, attachment to definite cellular macromolecules and stimulation for oxidative stress in cells (Akhtar, 2004).

Copper constitutes about 0.1% of earth's crust. It is an essential metal for many enzymatic reactions (Mehta *et al.,* 2006). Copper is a co-factor of various enzymes, including dehydrogenase, phosphatases, catalases, cytochrome C oxidase, peroxidase and superoxide dismutase and alcohol (MacPherson *et al.,* 2007). Low toxicity of Cu to mammals and terrestrial vertebrates was observed in 2005 by Uriu-Adams *et al.* (2005). The safe limit of copper for human being is 2 mg day^{-1} (Ariza *et al.,* 1999). It is extremely toxic to invertebrates, plants and microorganisms even at nano-molar concentrations ((Atienzar *et al.,* 2001; Babu *et al.,* 2001). Copper ion is usually a part of biological systems ((Achard *et al.,* 2007). Therefore, it leads to oxidative stress and formation of Reactive Oxygen Species (ROS) (Carter *et al.,* 1995). Production of reactive oxygen species due to Cu toxicity, induces the lipid peroxidation (Xie *et al.,* 2006), decreases the photosynthesis (Babu *et al.,*2001), causes the mitochondrial dysfunction (Arciello *et al.,* 2005) and destabilization of lysosomal membrane (Pourahmad *et al.,* 2004). In most bio-systems cellular protein's bound Cu ions are reduced by cellular antioxidants such as ascorbic acid, glutathione or O^{2-} (50, 42). This reduced metal may react with H_2O_2 to produce HO (Masad *et al.,* 2007). Hence hydrogen peroxide (H_2O_2) plays an important role in Cu toxicity (Kim *et al.,* 2007).

Sowing of legumes in cultivable soils has awesome effects on the fertility of soil (Chew, 2002). Presence of nodules on the roots of legumes has an additional ability of storing metals and thus hindering their translocation to edible part of the plant.

Phytoremediation may occur through phyto-extraction, phyto-stimulation, phyto-stabilization, phyto-transformation, phyto-volatilization, rhizofiltration and phytodegradation (Singh and Ward 2004). Biodegradation is meant by plant-microbe interaction, responsible for breakdown of organic contaminants in the rhizosphere (Euliss *et al.,* 2008). These processes may go together simultaneously or separately, depending on the target contaminants or the site conditions. Bio-remediation of metals by phytoextraction and phyto-stablization results into removal of metals or their sequesteration to lessen their exposure to the organisms (USEPA, 2000). Bio-oremediation in contaminated soil may be enhanced by plant growth promoting rhizobacteria (PGPR) which enable the plant to grow successfully in contaminated soil by better root development (Gambling *et al.,* 2004).

Heavy metals have well documented adverse effects on human health. These metals enter into environment from natural and anthropogenic sources. But later these contaminate the soil upto hazardous level. Plants in association with bacteria play important role in bioremediation of metal contaminated soils. Copper is an essential element for plant growth, but it becoms heavy metal beyond certain limits. However, there is very narrow range between its essentiality and harmfulness.

Rhizobacteria have great potential to play a role in the management of agricultural practices (Cook, 2002). In this regard, efficient strains of PGPR should fulfill at least two of three criteria, such as aggressive colonization, plant growth promotion and biocontrol (Vessey, 2003). Microbes, inhabiting in the rhizosphere may have negative, positive (Bashan *et al.,* 12) or neutral (Beattie, 2006) relationship with the plants (Whipps, 2001). Beneficial microbes may act as extracellular or intracellular PGPR (Martinze *et al.,* 2010). Endophytes like Allo-*Rhizobium*, Azo-*Rhizobium,* Brady-*Rhizobium*, Meso-*Rhizobium* and *Rhizobium* of the family rhizobiaceae reside within the nodules (Wang, 2000). They stimulate the plant growth either directly or indirectly.

The purpose of current study was to determine the capability of *Rhizobium* to help chickpea plant to grow successfully in metal contaminated soil.

MATERIALS AND METHODS

Isolation of *Rhizobium*

These studies were conducted in Soil Bacteriologty Sedtion, Ayub Agricultural Research Institute, Faisalabad, Pakistan during the year 2013-14 and 2014-15. *Rhizobium* was isolated from chickpea nodules. Nodules were sampled from different locations and dipped in 95% ethanol for four minutes, then rinsed consectively with sterile water and mercuric chloride solution (0.1% W/V). Subsequently, five washings were given to these nodules with sterile distilled water. The nodules were crushed with sterilized water in petri plates. Their juice was inoculated on the petri plates carried Yeast Mannitol Agar (YMA) media having congo red as an indicator. The plates were incubated at 28 ± 2ºC (Russell *et al.,* 1982). The pure white colonies (not attained the color of congo red), were re-streaked till the pure single colonies were obtained. The pure culture was kept at 4 ± 2º C and maintained for the experiment.

Characterization of isolates

Chemical characterization of isolates was carried out for auxin biosynthesis, oxidase test, catalase test, siderophore production and ACC-deaminase test (Table. 1). The strains of *Rhizobium* were tested for their auxin biosynthesis potential. The Yeast Mannitol Broth (YMB) was inoculated with *Rhizobium* isolates and incubated for 72 hours. The indole acetic acid equalants were determined using Salkowski's reagent (2 mL of 0.5 M $FeCl_3$ + 98 mL of 35% $HClO_4$) at 535 nm wavelength using spectrophotometer (Sarwar *et al.,* 1992). Oxidase test was performed to know the presence of oxidase enzyme in *Rhizobium* isolates (Steel, 1961). For this purpose, test culture was swabbed on an oxidase dry slide and color change into purple/blue was observed after one minute.

Table 1. Characterization of PGPR.

Isolated strains	Oxidase test	Catalase test	Siderophore	IAA* (µg mL^{-1})	ACC deaminase nmol g-1 biomass h-1
Isolate 1	+	+	+	8.0 a	161.22 b
Isolate 2	-	+	-	0.2 c	49.35 f
Isolate 3	-	+	+	0.6 c	54.33 e
Isolate 4	+	+	+	8.3 a	164.3 a
Isolate 5	+	+	-	6.6 b	160.3 b
Isolate 6	-	+	-	0.6 c	55.32 e
Isolate 7	+	-	-	0.4 c	86.66 d
Isolate 8	+	-	+	6.3 b	153.5 c
Isolate 9	+	+	+	7.4 ab	155.12 c
Isolate 10	+	+	-	0.3 c	53.22 e
LSD	-	-	-	1.1194	3.8463

IAA = Indole-3-acetic acid , ACC= 1-aminocyclopropane-1-carboxylic acid,

The PGPR isolates were evaluated for siderophore production on the chrome azurole S agar (CAS) as described by Clark and Bavoil (1994). Chrome azurole S agar plates were prepared and inoculated with test organism as spots.The inoculated plates were incubated at 30ºC for five days. Development of yellow-orange halo around the colony confirmed the production of siderophore.

ACC-deaminase activity was evaluted by monitoring the quantity of α-ketobutyrate produced by the rhizobacterial isolates due to hydrolysis of ACC, as described by Honma and Shimomura (1978). PGPRs were identified using the Biolog® identification system (Biolog™System Release 4.2, Hayward, CA, USA). Biolog® identification has been reported to be at par with the 16sRNA system (Vargas and Hara, 2006).

Screening of isolates

Eight treatments were included in this study; four treatments were uninoculated (control) and four were inoculated. A week before sowing, the pots were filled with sterilized sand and $CuSO_4$. $5H_2O$ @ 50, 100,150 and 200 mg Cu kg^{-1} soil, was added to all treatments. Chickpea seeds were inoculated by selected strains of peat based *Rhizobium* while control was inoculated with slurry of peat and Yeast Mannitol Broth. The growth promotion and nodulation were observed. Strains, showing good germination, root/shoot growth and nodulation were selected for experimentation.

Pot experiment

Pot experiment was conducted in two consecutive years. Medium textured soil was filled in pots, collected from farm area of Agriculture Biotechnology Research Institute, AARI, Faisalabad . Pre sowing soil analysis was done which showed pH 8.1, ECe 1.68 dSm^{-1}, nitrogen 0.027% and available P 7.5 mg kg^{-1}. Fertilizers @ 30- 60 NP kg ha^{-1} were applied to all the treatments. The experiment was arranged in completely randomized design with four replications. Treatments were as under:-

T_1 = Cu @50mg kg^{-1} soil(50 ppm)
T_2 = Cu @100mg kg^{-1} soil (100 ppm)
T_3 = Cu @150 mg kg^{-1} soil (150 ppm)
T_4 = Cu @200 mg kg^{-1} soil (200 ppm)
T_5 = T_1+*Rhizobium* inoculation
T_6 = T_2+*Rhizobium* inoculation
T_7 = T_3+*Rhizobium* inoculation
T_8 = T_4+*Rhizobium* inoculation

Crop was closely monitored during the entire growth period. Weeding and plant protection measures were adopted as per requirement. At flowering (50 days after sowing) data on root parameters (number of nodules, nodular mass, root length and root dry weight) were recorded. Other parameters were determined after harvest. Samples were oven-dried at 65ºC for 48 hours, prior to take dry weight. Soil was analysed for phosphorus contents by modified Olsen method (Olsen and Sommers, 1982) while Cu contents in the soil were determined by Atomic Absorption Spectrophotometer (Ezell, 1967).

Statistical analysis

Data were subjected to statistical analysis following CRD using standard procedures (Steel *et al.,* 1997). The difference among the treatment means was compared by applying the Duncan's multiple range tests (Duncan, 1955).

RESULTS AND DISCUSSION

Data regarding presowing soil analysis were recorded ten days after the incubation of $CuSO_4.5H_2O$ (Table 2). The results indicated that available P at 50 and 100 mg kg^{-1} Cu was higher compared to 150 and 200 mg $kg^{-1}$of Cu. However, there was a great variation in Cu contents at 50 mg kg^{-1} Cu (42.5 ppm) to 200 mg kg^{-1} Cu (156.7 ppm).

Table 2. Pre-sowing status of Cu and P in soil mean of three repeats.

Treatments	*Pre sowing soil Cu (ppm)	*Pre sowing soil P (ppm)
T_1 = Cu @ 50 mg kg^{-1}	42.5d	7.07ab
T_2 = Cu @ 100 mg kg^{-1}	69.7c	7.14a
T_3 = Cu @ 150 mg kg^{-1}	93.7b	6.74c
T_4 = Cu @ 200 mg kg^{-1}	156.7a	6.05d
LSD	5.0244	0.4629

*Samples were collected 10 days after incubation of $CuSO_4.5H_2O$
Means sharing similar letter(s) in a column do not differ significantly at p<0.05 according to Duncan's multiple range test.

Rhizobium inoculated to chickpea showed positive effects on the root parameters of chickpea (Table 3). These effects were more pronounced when inoculants were applied at 50 ppm Cu level (T_5) giving highest number of nodules (35.0) and nodular mass (0.19 g $plant^{-1}$) followed by 100 ppm Cu level (32 and 0.18 g $plant^{-1}$) (T_6). Lowest nodular number and mass (24 and 0.11 g $plant^{-1}$) were observed at 200 ppm level of Cu (T_4).

Legumes, as food item, have an edge over other crops since these accumulate the metals in their nodules and let least amount of metal to be translocated in the edible parts. The signal exchange between *Rhizobium* and legumes is responsible for nodulation (Perret *et al.,* 2000). Nodules present on the legume roots are increased in number with the application of appropriate strain which in turn enhances the root length, mass

Table 3. Effect of *Rhizobium* inoculation on the root parameters of chickpea mean of three repeats.

Treatments	No.of nodules plant^{-1}	Nodular mass (g plant^{-1})	Root length (cm)	Root dry weight (g plant^{-1})
T_1 = Cu @50mg kg^{-1}	31 c	0.16 bc	35.0 b	1.7 bc
T_2 = Cu @100mg kg^{-1}	29 cd	0.15 cd	33.0 c	1.6 bc
T_3 = Cu @150 mg kg^{-1}	27 e	0.12 e	26.3 d	1.3 de
T_4 = Cu @200 mg kg^{-1}	24 f	0.11 e	21.0 f	1.1 e
T_5 = T_1+*Rhizobium* inocu.	35 a	0.19 a	38.0 a	2.1 a
T_6 = T_2+*Rhizobium* inocu.	32 b	0.18 ab	35.0 b	1.8 b
T_7 = T_3+*Rhizobium* inocu.	29 de	0.16 bc	26.3 d	1.6 c
T_8 = T_4+*Rhizobium* inocu.	28 de	0.13 de	23.0 e	1.3 d
LSD	1.7123	0.0262	1.7975	0.1756

Means sharing similar letter(s) in a column do not differ significantly at p<0.05 according to Duncan's multiple range test.

and efficiency of nodules. In present study, the root parameters were adversely affected by increasing the concentration of Cu metal as compared to the control. Singla and Garg (2005) have also reported that chickpea roots showed more sensitivty to metal stress than shoots. However, rhizobial inoculation enhanced the nodulation compared to their respective control. Present results are at par with the findings of Huang and Erickson (2007) that inoculation with *Rhizobium* increased the nodulation in lentil and peas as well as in peanut (Dey *et al.* 2004). Nodular mass was also increased due to inoculation even under metal stress condition (Kiros *et al.,* 2007). They further observed that nodular number and nodular mass were increased in peas by *Rhizobium* inoculation. Nodule dry weight prove to be an important parameter to indicate the effective symbiosis (Ogutcus *et al.,* 2008).

The significant increase in root length (38 cm) and root dry weight (2.1 g plant^{-1}) was observed in *Rhizobium* inoculation at 50 ppm Cu concentration, followed by 100 ppm Cu level plus *Rhizobium* inoculation and this was statistically at par with the 50 ppm Cu treatment without inoculation (Table 3). At higher concentration of Cu (150 ppm) root length (26.3 cm) was not significantly affected by inoculation. It was further observed that root length was decreased with the increased concentration of Cu but there was a significant increase in root length by inoculation with *Rhizobium*. These findings are in line with those of Albayrak *et al.* (2006) who concluded that *Rhizobium* inoculation had better effects on the growth and yield of *Vicia sativa* L. Seed inoculated plants, exhibited increased root and shoot length as compared to un-inoculated control (Ali *et al.,* 2008; Khaleuzzaman and Hossain, 2007). In this study root weight also decreased with the increased concentration of metal and increased with inoculation compared to control. Similar results were reported by Zarrin *et al.* (2006) who observed that *Rhizobium* enhanced root/ shoot dry and fresh weight of inoculated soybean.

Rhizobium effect on yield parameters

The results (Table 4) indicated that Cu upto 50 ppm concentration caused no significant recession, in yield parameters, due to rhizobial inoculation. Significantly higher shoot length (34.7cm) was observed by *Rhizobium* inoculation with 50 ppm Cu contents followed by 100 mg cu (32.4). Shoot length by *Rhizobium* inoculation at 200 ppm cu contents (26.5 cm)was statistically at par with the 150 ppm Cu without inoculation (Compensation of 50 ppm Cu contents). Similar trend was observed in shoot dry weight.

Table 4. Effect of *Rhizobium* inoculation on the yield parameters of chickpea mean of three repeats.

Treatmants	Shoot length (cm)	Shoot dry weight (g)	Biomass (g plant^{-1})	No. of pods plant^{-1}	Grain yield (g plant^{-1})
T_1 = Cu @50mg kg^{-1}	30.6 c	2.4 b	27.8 b	15.3 bc	14.3 b
T_2 = Cu @100mg kg^{-1}	27.0 d	1.9 c	24.4 c	13.6 de	13.2 b
T_3 = Cu @150 mg kg^{-1}	23.9 e	1.6d e	20.8 d	11.4 f	12.0 c
T_4 = Cu @200 mg kg^{-1}	22.2 f	1.40 e	18.3 e	10.8 f	11.0 c
T_5 = T_1 + *Rhizobium* inocu.	34.7 a	2.80 a	31.4 a	17.0 a	16.8 a
T_6 = T_2 + *Rhizobium* inocu.	32.4 b	2.50 b	30.9 a	16.4 ab	16.1 a
T_7 = T_3 + *Rhizobium* inocu.	26.5 d	1.90 c	24.5 c	14.5 cd	14.3 b
T_8 = T_4 + *Rhizobium* inocu.	22.2 f	1.8 cd	21.2 d	12.4 ef	11.9 c
LSD	1.4364	0.1291	1.9117	1.6479	1.1242

Means sharing similar letter(s) in a column do not differ significantly at p<0.05 according to Duncan's multiple range tests

Inoculated treatment at 100 ppm Cu was statistically at par with 50 ppm Cu without inoculation. Shoot length as well as shoot dry weight decreased with the increased Cu contents but *Rhizobium* inoculation at Cu contents of 100 ppm kept shoot length (32.4cm) and weight (2.5g plant^{-1}) statistically at par with that of un-inoculated treatment (30.6 cm, 2.4 g plant^{-1}) having 50 ppm Cu contents. Singla and Garg (2005) also observed decreased root and shoot weight of chickpea under stress condition. Shoot dry weight was directly proportional to the nodular mass and same was also reported by Ballara *et al.* (2004).

The data (Table 4) further indicated that biomass was not significantly reduced by increasing Cu contents from 50 ppm (31.4 g plant^{-1}) (T_5) to 100 ppm (30.9 g plant^{-1}) (T_6) due to *Rhizobium* inoculation. Biomass is a dependent variable, more the shoot and grain mass the more will be the total biomass. Chemining'wa *et al.,* (2012) reported that biomass was also directly proportional to the number of nodules. Pods per plant and accordingly the grain yield was reduced by increasing Cu concentration. However, inoculation with *Rhizobium,* significantly increased the given parameters. The positive response of legume crop by *Rhizobium* inoculation was also reported by Yesim *et al.* (2008).

Compensation of 50 ppm Cu was also observed by *Rhizobium* inoculation in biomass. It was 24.5 g plant^{-1} at 150 ppm Cu due to *Rhizobium* inoculation (T_7) which was statistically at par with Cu contents of 100 ppm without inoculation (24.4 g plant^{-1}) (T_2). Number of pods (17.0 16.4) and grain yield (16.8, 16.1 g plant^{-1}) at Cu contents of 50 and 100 ppm, respectively, was significantly higher by *Rhizobium* inoculation compared to all other treatments. Grain yield (14.3 g plant^{-1}) at 150 ppm Cu with *Rhizobium* inoculation (T_7) was statistically at par with 50 ppm (14.3 g plant^{-1}) and 100 ppm Cu (13.2 g plant^{-1}) without inoculation (T_2)

Soil analysis

Data regarding post-harvest soil analysis (Table 5) revealed that inoculation of *Rhizobium* enhanced available P at all levels of Cu contents as compared to control. Maximum available P (12.17 mg kg^{-1}) was recorded at 50 ppm Cu concentration with rhizobial inoculation (T_5) compared to its respective control (9.67 ppm) (T_1).

Phosphorus contents were increased in the post harvest soil samples. Phosphorus availability was increased with the decrease in pH, down to slightly acidic range (6.5). Khan *et al.,* (2006) inferred that microbial population produces organic acids which acidifies the organic/microbial cell and its surroundings. Hence the release of protons during N_2-fixation by *Rhizobium* and the production of organic acids reduce the soil pH which might be the possible mechanism for pH reduction.

Table 5. Post harvest contents of Cu and P in the rhizosphere mean of three repeats.

Treatments	Post harvest soil Cu (ppm)	Post harvest soil P (ppm)
T_1. Cu @ 50mg kg^{-1}	37.5d	9.67c
T_2. Cu @ 100mg kg^{-1}	43.8c	9.4cd
T_3. Cu @ 150 mg kg^{-1}	61.6b	8.94cd
T_4. Cu @ 200 mg kg^{-1}	84.1a	8.04e
T_5 = T_1+*Rhizobium* inocu	24.5f	12.17a
T_6 = T_2 + *Rhizobium* inocu	30.5e	1043b
T_7 = T_3 + *Rhizobium* inocu.	47.3c	9.29cd
T_8 = T_4 + *Rhizobium* inocu.	63.1b	8.89d
LSD	5.9891	0.7629

Means sharing similar letter(s) in a column do not differ significantly at p<0.05 according to Duncan's multiple range test

Under Pakistani conditons, pH remains above 8, therefore, the production of organic acids favours the P availability. Suneja *et al.* (2007) also reported the pH dependent nutrient availability. However, there was slight increase in post harvest P contents than pre-sowing P while Cu contents were decreased from 156.7 ppm to 63.1 ppm in post harvest samples by the application of rhizobial inoculum. Copper contents of soil were decreased from 73 to 99% by the application of *Rhizobium* inoculum. It was due to the fact that plant uptake macro as well as micronutrients from the soil and siderophore producing bacteria chelates the micronutrients from the soil and make them available to plant (Burd *et al.,* 2000). Rhizobacteria release organic acids thus enhance the uptake of metals by the plant (Abou Shenab *et al.,* 2003) and transform the heavy metals into the form which the plant can uptake safely (Zayed *et al.,* 1998). *Rhizobium* species act as phytoextraction assistants (Abou Shanab *et al.,* 2006). Plants consume the required quantity and accumulate the excess in different body parts i.e. nodules, root, stem, leaves and grain.

CONCLUSION

Rhizobium helps in bio-remediation of metal contaminated soils. Deleterious effects of heavy metals may be controlled either by enhancing the metal storing capability of plants or by increasing the plant biomass. Rhizo bacterial inoculation to the plants in metal contaminated soils may promote their growth and increase their biomass by encouraging re-vegetation and supporting remediation. Use of rhizobateria in association with plants can provide high efficiency for bio-remediation yet there is a long way to fully explore the microbial ecology in the rhizosphere.

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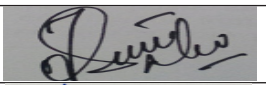
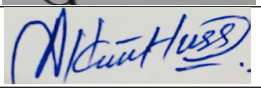
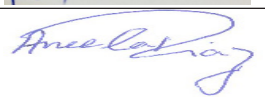
CONTRIBUTION OF AUTHORS

S. No.	Name of author	Contribution	Signatures
1.	Naseem Akhtar	Conducted research trial and wrote paper	
2.	Azhar Hussain	Reviewed the relevant literature	
3.	Aneela Riaz	Isolated Bacterial strains purified and prepared inoculums for the experiment.	
4.	Muhammad Aftab	Analysed research samples