Effects of nanocrystalline powders (Fe, Co and Cu) on the germination, growth, crop yield and product quality of soybean (Vietnamese species DT-51)

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Received 14 November 2013
Accepted for publication 29 January 2014
Published 28 February 2014

Abstract
Superdispersive iron, cobalt and copper nanocrystalline powders were synthesized in a water–ethanol medium by the reduction method using sodium borohydride as a reducing agent and carboxymethyl cellulose as a stabilizer (for Fe and Co nanoparticles). Transmission electron microscopy micrographs and x-ray diffraction analyses of the freshly prepared nanocrystalline powders indicated that they were in a zerovalent state with particle sizes ranging from 20 to 60 nm. The soybean seeds were treated with an extra low nanocrystalline dose (not more than 300 mg of each metal per hectare) and then sowed on an experimental landfill plot consisting of a farming area of 180 m². This pre-sowing treatment of soybean seeds, which does not exert any adverse effect on the soil environment, reliably changed the biological indices of the plant growth and development. In particular, in laboratory experiments, the germination rates of soybean seeds treated with zerovalent Cu, Co and Fe were 65, 80 and 80%, respectively, whereas 55% germination was observed in the control sample; in the field experiment, for all of the nanoscale metals studied, the chlorophyll index increased by 7–15% and the number of nodules by 20–49% compared to the control sample, and the soybean crop yield increased up to 16% in comparison with the control sample.

Keywords: nanoscale zerovalent, metal powder, germination, soybean, seed

Classification numbers: 1.00, 2.03, 2.04, 4.02, 4.04

1. Introduction
In recent decades, nanotechnology products have been intensively applied in agriculture. Nanocrystallines are known as a stimulating agent for plant growth and the activation of metabolic processes in plant and animal organisms. Numerous researchers have studied the biological effects of nanoparticles, as well as their beneficial and harmful effects on plants. Observing the seed germination and seedling growth of Boswellia ovalifoliolata (an endemic and globally threatened medicinal tree species) in the presence of silver nanoparticles with a concentration of 10 μg ml⁻¹ and particle size of 30–40 nm, Savithramma et al [1] showed that the germination rate of seeds treated with silver nanoparticles was 28% higher than that of the control sample and the seedling height doubled, while the sprouting of seeds was observed on the seventh day of silver nanoparticles’ treatment compared to the 15th day of the control sample. According to the authors, the contribution of nanoparticles was to facilitate the motion of water and nutrients through a seed coat for

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accelerating the seed germination and seedling growth of
the plant.

Lu et al [2] studied the effect of nano-SiO₂ and
nano-TiO₂ mixtures on soybean seeds and found that the
mixture increased the nitrate reductase in soybeans, increasing
their germination and growth. Mahajan et al [3] reported
the effects of ZnO nanoparticles on the growth of mung
(Vigna radiata) seedlings, the seeds of which were previously
allowed to germinate in wet cotton for 24 h in the dark,
then the sprouted seeds were taken for further study of
the seedlings which grew in culture media containing
nanoparticles. The authors found the maximum growth effect
with a nano-ZnO concentration of 20 ppm and beyond this
value the seedling growth was inhibited, which might be
attributed to the toxic level of nanoparticles. The effect of
nanoparticles ZnO, FeO and ZnCuFe-oxide on the growth
of mung seedlings was investigated by Dhoke et al [4]. The
study was carried out by spraying different concentrations of
nanoparticles in suspension form on hydroponically grown
test units.

According to a review [5], silver nanoparticles and
nanocrystalline oxides ZnO and CuO have been studied in
agriculture due to their ability to restrict bacterial growth.
The study focused on the interaction of these nanoparticles
with beneficial bacteria, particularly on the responses of
psuedomonads as these microbes are found globally in
soils and are major players in plant growth and in soil
carbon and nitrogen cycling. Using microscopy tools and
techniques to explore the benefits of applying nanotechnology
to agriculture, Gonzalez-Melendi et al [6] visualized and
tracked the transport and deposition of magnetic nanoparticles
inside the plant, and checked the possibility of concentrating
the nanoparticles into localized areas. The authors showed
that with the aid of a magnet iron, nanoparticles could be
concentrated in certain areas in a pumpkin plant. Prasad et al [7]
investigated the promotory and inhibitory effects of
ZnO nanoparticles (mean particle diameter of 20 nm) on
growth and final yield of groundnut. The result showed that
peanut seeds treated with a nano-ZnO suspension of 1000 ppm
(concentration corresponding to a dose of about 20 g ha⁻¹)
promoted both seed germination and seedling vigor. The
nanoparticles proved to be effective in increasing stem and
root growth, while an inhibitory effect was found at a
concentration of 2000 ppm.

The combined effects of silver nanoparticles and
permanent magnetic field on growth of fodder maize were
studied by Berahmand et al [8]. After seed emergence,
magnetic treatment was performed by employing magnet
pieces with a strength of 10 mT, located adjacent to each
plant on the soil’s surface. Then 40 g ha⁻¹ of colloidal
nanosilver was used for irrigation. As a result, the maize
dry matter yield increased by 23% compared to the control
sample.

Obviously, most of the aforementioned works dealt with
moderate metal concentrations, which, like microfertilizers in
salt or chelate form, may result in the accumulation of heavy
metals in the soil environment [9]. Meanwhile, limited studies
have been reported on the promotory effects of nanoparticles
on plants which underwent pre-sowing treatment of seeds
with extra low nanoscale metal concentrations. Biogenic
nanocrystallines such as Fe, Mn, Zn, Cu, Co, Se, etc,
among which Fe, Cu and Co with variable valences are the
most bioactive [10], have been widely used in agriculture as
they actively participate in different redox processes in
plants and are present in the composition of many enzymes
and complicated proteins [11–15]. A distinctive feature of
metal nanoparticles, so-called superdispersive metal powders
(SDMP), was the low toxicity compared to their salts and
chelates, and the ability to stimulate physiological and
biochemical processes in plants by using their extra small
doses in pre-sowing treatment of seeds [12, 15]. Polischuk
and co-workers [10] showed that treatment of seeds by SDMP
before sowing may increase the protein content up to 40%,
depending on the metal nanopowder used. Before sowing
the seeds were treated with a sonicated aqueous SDMP
suspension of different concentrations (20–80 mg ha⁻¹), while
the value of these concentrations in turn depended on the
plant species and metal powder used [13, 14]. Sonication of
SDMP before treatment of seeds is a crucial moment, as
according to the work [12], SDMP in their pure form
do not exhibit biological activity, but in aqueous solution
after ultrasonic treatment the active atoms appearing on
their surface will be oxidized, giving free electrons. On the
other hand, it was found that the effect of SDMP on seeds
during pre-sowing treatment begins with the respiration and
sprouting processes, where in the foreground stand hydrogen
and molecular oxygen, which need free electrons for carrying
out their exchange reactions [14]. In other words, SDMP is an
additional source of free active electrons used for stimulating
metabolic processes.

The maximum protein content was found when seeds were
treated with cobalt nanoparticles (30–60 mg ha⁻¹), which
actively affects the nitrogen exchange followed by
stimulating protein synthesis. When vetch seeds (from a
herbaceous plant) were coated with copper nanoparticles
before sowing, the content of ascorbic acid increased by
approximately three times, while that of carotene increased
four times in comparison with the control sample, depending
on the plant development phase [10].

In this report, the effects of nanocrystalline Fe, Co
and Cu at extra low doses on germination, growth, crop
yield and product quality of soybeans were studied using the
SDMP produced by the aqueous chemical reduction method.

2. Materials and methods

Nanocrystalline iron, cobalt and copper with sizes from
20 to 60 nm were produced following the aqueous
solution reduction method with sodium borohydride as
a reducing agent and carboxymethyl cellulose (CMC) as
a stabilizer [16–21]. FeSO₄ 7H₂O, CoSO₄ 7H₂O, CuSO₄
5H₂O, NaBH₄ and CMC were purchased from Merck
Chemical Reagent Co.; C₂H₅OH (absolute) from Beijing
Chemical Reagent Co. For laboratory experiment, plant agar
(Duchefa Co.) and nutrient solution MS (Murashige and
Skog medium) were used. Protein, lipid and sugar contents
were determined according to Kjeldahl, Randall and Lane
and Eynon methods [22], while minerals were determined
according to the association of official analytical communities
(AOAC) method [23].
2.1. Preparation of iron, cobalt and copper metal nanoparticles

For preparing 1 g of iron nanoparticles, 18 mmol of FeSO$_4$ 7H$_2$O was dissolved in 160 ml deionized water and stirred for 10 min in a 500 ml flask, and 40 ml of 0.5% CMC solution was added to the flask and vigorously stirred (1500 rpm) for 15 min. Then, without disrupting stirring, 2 g of NaBH$_4$ was poured into the mixture and the stirring was continued for another 10 min. The precipitate was separated from the supernatant by using a permanent 0.45 T magnetic field. The zerovalent metal (ZVM) nanoparticles obtained were washed thoroughly (not less than five times) with absolute ethanol in order to maximally remove accompanied ions and then vacuum-dried at 70 $^\circ$C for obtaining the final product in a powder form.

Cobalt ZVM nanoparticles were prepared by the same procedure, except that 1 g of NaBH$_4$ was used instead of 2 g for iron preparation, as its self-oxidation capacity is much weaker than that of the latter.

In a typical procedure (figure 1) for preparing 1 g of copper metal nanoparticles, 15.62 mmol of CuSO$_4$ 5H$_2$O was added to 50 ml deionized water in a 200 ml flask and vigorously stirred for 10 min to dissolve the salt completely. Without disrupting stirring, 1.2 g of NaBH$_4$ was poured into the flask and the stirring was continued for another 10 min. The precipitate was separated from the supernatant by centrifugation (rotation speed of 9000 rpm). As long as the zerovalent copper nanoparticle was able to form a thin oxide layer film on its surface, there was no need to use a capping agent. To remove accompanied ions, the precipitate was washed thoroughly with absolute ethanol (not less than five times) using centrifuge for precipitates' separation. Then the purified precipitate was vacuum-dried overnight at 70 $^\circ$C to obtain the powdery copper metal nanoparticles. The obtained nanocrystalline metals in the form of superdispersive powders were stored in hermetic flasks filled with argon gas.

2.2. Soybean seed treatment

Soybean seeds of Vietnamese species DT-51 were procured from Legumes Research and Development Center of Field Crop Research Institute, Vietnam Academy of Agricultural Sciences. Treatment of the seeds with nanoparticles was carried out as follows (figure 2): a defined amount of each ZVM nanopowder was dispersed in a closed polyethylene (PE) pocket with a certain water volume, sonicated (800 W, 20 kHz) for 30 min and soybean seeds were immersed in this suspension for 45 min. After being removed from the PE pocket and dried in air, the treated seeds were ready (within 24 h) for sowing experiments.

2.3. Laboratory experiment

One set of germination experiments was carried out in laboratory for determining the treatment effect on seed germination and seedling vigor. Soybean seeds were sterilized in a desiccator containing 0.5% sodium hypochlorite solution for 30 min to ensure surface sterility and rinsed thoroughly with deionized water. To investigate the promotory and inhibitory effects of nanoparticles on soybean plant growth, three concentrations of ZVM nanopowders were used: 0.080, 0.200 and 0.320 g ha$^{-1}$, respectively. The nanocrystalline-treated seeds were immediately transferred to Petri dishes, each of which contained 80 ml of 7.5% agar solution, for growing up to 5 days. The arrangement of set I consisted of three columns of Petri dishes for three nanocrystallines and three rows for three nanocrystalline concentrations, respectively, and one row (two dishes) for the control (eight replicates). For seed germination Petri dishes were sprayed with nutrient solution every day. Five days after sowing the soybean plants were uprooted and the total length of roots and shoots were measured.

2.4. Field experiment

The field experiments involved three Vietnamese iron, cobalt and copper nanopowders and one Russian nanocopper, which
was produced by the low-temperature reduction method [15]. The experiments were conducted on an experimental landfill plot at Hanoi Agricultural Promotion Center, in randomized complete block design, replicated six times. The total useful plot size 180 m² was divided into 30 compartments ((4trials + 1control) × 6 m² per compartment × 6replicates). Chemical characteristics of the soil from the experimental landfill plot were determined in the Environmental Chemistry Laboratory, Institute of Chemistry, Vietnam Academy of Science and Technology (table 1). Before sowing soybean seeds were treated with one of the zerovalent metal nanoparticles with a dose of 0.080 g ha⁻¹.

3. Results and discussion

3.1. Characterization of the SDMP

Transmission electron microscopy (TEM) micrographs of nanocrystalline iron, copper and cobalt prepared by the aqueous solution reduction method (figure 3, upper row) showed that mean particle sizes ranged from 20 to 60 nm. As aforementioned, for use in plant cultivation, metal nanoparticles should be principally in the zerovalent state. Therefore, the preparation process of SDMP requires the use of a capping agent to restrict them from oxidation. For this aim, CMC was chosen as a stabilizing agent partially due to its adequate solubility in water. In our work, CMC was used for preparation of Fe and Co SDMP, while for copper SDMP was prepared without using a capping agent, because nanocrystalline copper metal formed a thin firm oxide layer on its surface. On the scanning electron microscope (SEM) images presented on the lower row (figure 3) one can see that nanocrystalline copper possessed the smallest particle size.

Table 1. Chemical characteristics of the soil taken from the experimental landfill plot.

| No. | Parameters         | Values          |
|-----|--------------------|-----------------|
| 1   | pH                 | 7.82            |
| 2   | Total nitrogen     | 2.04 mg g⁻¹ dry |
| 3   | Total phosphor     | 0.67 mg g⁻¹ dry |
| 4   | Total organic carbon | 68.1 mg g⁻¹ dry |
| 5   | Kali (K)           | 3.40 mg g⁻¹ fresh |
| 6   | Iron (Fe)          | 32.21 mg g⁻¹ dry |
| 7   | Copper (Cu)        | 10.65 mg kg⁻¹ fresh |
| 8   | Cobalt (Co)        | 2.32 mg kg⁻¹ fresh |
Figure 4. Influence of washing cycles on the SDMP purity. (a) Iron and (b) cobalt SDMP washed four times, respectively, (c) copper SDMP washed six times with absolute ethanol.

Figure 5. Growth inhibiting effect of nanoscale ZVM doses.

It may be due to the fact that the reaction of nanoparticle formation without a capping agent occurred with a higher speed of appearance of crystalline centers, resulted in the formation of smaller particles compared to iron and cobalt nanoparticles which were covered with layers of CMC.

Biological activity of SDMP strongly depended on their purity, because surface energy might be lost in the presence of ions on their surface [11, 15]. The purity degree of SDMP depends upon the washing cycles. After four washing cycles with absolute ethanol the content of sulfur on Fe and Co SDMP remained remarkable (figures 4(a) and (b), but for Cu SDMP after six cycles the sulfur content diminished considerably (figure 4(c)).

3.2. Seed germination and seedling vigor (laboratory experiment)

Soybean seeds responded differently toward the pre-sowing treatment at various concentrations of the SDMP and toward different nanocrystalline metals. The effect of nanocrystalline Fe, Cu and Co on soybean germination and vigor depicted in table 2 and figure 5 showed that a SDMP dose of 0.08 g ha\(^{-1}\) demonstrated the best result in germination and seedling vigor compared to the higher doses. Among the three metals studied, nanocrystalline cobalt gave the best seedling vigor index (SVI) value, exceeding 2.4 times that of the control sample. This seedling vigor value was comparable with that in the work [7], in which peanut seeds were growing in an agar medium in the presence of ZnO nanoparticles.

The experimental data presented in figure 5 proved that increasing the SDMP doses exceeding 0.080 g ha\(^{-1}\) could inhibit the germination rate and seedling vigor. Among the three nanocrystalline metals tested nanocopper demonstrated the strongest inhibitive effect on germination and seedling vigor (table 2, figure 6): at Cu doses 0.20 and 0.32 g ha\(^{-1}\) germination rates decreased by 0 and 9.1% compared to the control sample, respectively, while SVI decreased 5.8 and 41.2% compared to SVI of the dose of 0.08 g ha\(^{-1}\). These inhibition effect results of SDMP doses were in good agreement with other researchers [7, 10, 14] and implied a possibility to minimize the optimal doses toward smaller values.

Analytical results of biochemical composition of nanocobalt-treated soybean sprouts after 5 days growing...
Table 2. The effect of nanoscale ZVM Cu, Fe and Co on soybean germination and vigor (laboratory experiment).

| Nanocrystallines | Germination (%) | Root length (cm) | Shoot length (cm) | SVI a |
|------------------|-----------------|------------------|-------------------|-------|
| Control          | 55 ± 4.7        | 0.66 ± 0.25      | 3.37 ± 0.48       | 222.04 ± 0.27 |
| Cu               | 65 ± 5.3        | 0.73 ± 0.14      | 4.12 ± 0.52       | 314.73 ± 0.22 |
| Fe               | 80 ± 5.6        | 1.35 ± 0.37      | 4.80 ± 0.39       | 491.68 ± 0.19 |
| Co               | 80 ± 4.3        | 1.26 ± 0.25      | 5.30 ± 0.38       | 524.56 ± 0.15 |
| Cu               | 55 ± 6.1        | 0.96 ± 0.19      | 4.44 ± 0.58       | 296.50 ± 0.25 |
| Fe               | 70 ± 5.6        | 0.74 ± 0.12      | 5.22 ± 0.39       | 416.99 ± 0.17 |
| Co               | 61 ± 6.3        | 0.98 ± 0.28      | 4.81 ± 0.28       | 353.13 ± 0.20 |

Nanocrystalline dose = 0.08 g ha⁻¹

Nanocrystalline dose = 0.20 g ha⁻¹

Nanocrystalline dose = 0.32 g ha⁻¹

Cu 50 ± 5.86 b 0.53 ± 0.14 3.18 ± 0.40 185.50 ± 0.26
Fe 60 ± 6.24 b 1.70 ± 0.61 4.79 ± 0.81 389.34 ± 0.32
Co 56 ± 5.47 b 0.81 ± 0.16 5.12 ± 0.49 332.50 ± 0.18

a Seedling vigor index (SVI) = germination (%) × (root length (cm) + shoot length (cm)).

b p ≥ 0.05

Figure 6. Inhibition effect of various nanocrystalline powder doses on germination and growth of the treated soybean seeds.

Figure 7. Crop yield of soybean, the seeds of which were treated with SDMP.

(table 3) showed that all biochemical parameters actually remained the same as those in the control sample, although protein and calcium contents were reliably higher and those of total sugar and zinc were lower than that in the control sample (p ≤ 0.05). These results correlated with those of other works [10, 12, 15].

3.3. Field experiment

Results of the field experiment (table 4, figures 7 and 8) confirmed that, just as in laboratory experiments, cobalt nanopowder exhibited a better germination effect than nanoscaled iron and copper, wherein all the growth parameters exceeded the control ones (with crop yield surpassed the control one by 16%). In field experiments, an additional trial set was conducted on a Russian copper nanopowder, which

Figure 8. SDMP-treated soybean (hybrid species DT51) in the experimental field: (a) in vegetative stage and (b) in crop stage.

Table 3. Biochemical composition of the nanocobalt-treated soybean seeds after 5 days cultivation (Vietnamese species DT-51).

| Composition                                  | Control       | Co-treated   |
|----------------------------------------------|---------------|--------------|
| Protein (g per 100 g) a                       | 31.59 ± 0.63  | 33.52 ± 0.67 |
| Lipid (g per 100 g)                           | 19.62 ± 0.39  | 19.66 ± 0.40 |
| T. sugar (g per 100 g) a                      | 17.48 ± 0.35  | 16.2 ± 0.32  |
| Fe (mg per 100 g) b                           | 9.4 ± 0.2     | 9.87 ± 0.20  |
| Ca (mg per 100 g)                             | 496.3 ± 10    | 514 ± 10     |
| Mg (mg per 100 g)                             | 258 ± 5.4     | 260 ± 6      |
| K (mg per 100 g)                              | 1340 ± 26     | 1370 ± 26    |
| Cu (mg per 100 g)                             | 1.85 ± 0.04   | 1.83 ± 0.04  |
| Zn (mg per 100 g)                             | 5.28 ± 0.10   | 4.93 ± 0.10  |

a p ≤ 0.05.
was produced by the low-temperature hydrogen reduction method (table 4, fourth column). The data showed that all the growth parameters of both the experimental group of ‘Vietnamese’ copper and the ‘Russian’ one were almost the same. This result additionally proved that SDMP produced by the aqueous chemical reduction method possessed similar biological activity as those produced by the low-temperature hydrogen reduction method and could be used for agricultural application purposes.

4. Conclusion

Nanocrystalline Fe, Co and Cu with particle sizes ranging from 20 to 60 nm have been synthesized by the aqueous chemical reduction method using sodium borohydride as the reducing agent and CMC as the stabilizer to prevent oxidation of iron and cobalt ZVMs.

Nanocrystalline metal-treated soybean seeds responded in various ways to different SDMP concentrations and to different nanocrystalline metals: among the SDMP studied, nanocrystalline cobalt exhibited the best biological effects on soybean growth and development, with the SVI surpassing that of iron and cobalt ZVMs.

SDMP produced by the aqueous chemical reduction method with extra low doses can be used for agricultural application.

Acknowledgment

This research was financially supported by Vietnam Academy of Science and Technology.

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Table 4. Basic growth parameters and crop yield of nanocrystalline-treated soybean (Vietnamese species DT-51). Nanocrystalline powder dose: 0.08 g ha⁻¹.

| SDMP       | Control | Cu    | Cu^a | Fe   | Co   |
|------------|---------|-------|------|------|------|
| Chlorophyll content (mg per 100 g leaf⁻¹) | 27.0 ± 0.9 | 29.1 ± 0.6 | 28.6 ± 0.7 | 31.7 ± 0.8 | 29.6 ± 0.8 |
| Number of nodules/root | 13.1 ± 1.6 | 19.7 ± 4.4 | 16.2 ± 6.1 | 12.9 ± 8.7 | 16.8 ± 7.0 |
| Number of pods/plant | 76.2 ± 16.4 | 81.1 ± 18.1 | 80.3 ± 12.9 | 63.6 ± 16.1 | 89.1 ± 18.6 |
| Pods weight (g)/plant | 63.7 ± 14.4 | 57.0 ± 12.9 | 60.3 ± 17.4 | 52.1 ± 23.1 | 72.1 ± 15.4 |
| Weight of 1000 grains (g) | 162.2 ± 3.1 | 169.2 ± 2.8 | 161.9 ± 8.5 | 162.2 ± 2.9 | 166.0 ± 5.8 |
| Crop yield (ton ha⁻¹) | 2.33 ± 0.06 | 2.59 ± 0.08 | 2.46 ± 0.06 | 1.95 ± 0.05 | 2.71 ± 0.08 |

^a Russian copper nanocrystalline powder, produced by the low-temperature hydrogen reduction method.