Antioxidant capacity and germination power of NaCl-elicited cowpea (Vigna unguiculata) sprouts with various NaCl concentrations and elicitation durations

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Abstract. Cowpea (Vigna unguiculata) is one of legumes that have the potential as a source of food antioxidant related to the phenolic compounds. Germination can enhance the antioxidant capacity of cowpea and elicitation using NaCl within the germination process. It has been reported as an effective technique to improve the antioxidant potential of legumes sprouts. This study aimed to evaluate the antioxidant capacity (total phenolic content, DPPH radical scavenging activity, and reducing power (RP)) of NaCl-elicited cowpea sprouts with different concentrations of NaCl (50, 100, 150 mM) and elicitation durations (8, 12, 16 hours). The germination power of the NaCl-elicited cowpea was also investigated. Results showed that the total phenolic content, DPPH radical scavenging activity and RP of NaCl-elicited cowpea sprouts increase in line with the increase in NaCl concentrations and elicitation durations. Both DPPH radical scavenging activity and RP had significant correlation (p<0.01; r = 0.805; 0.785) with the total phenolic content. Nevertheless, germination power decreased along with increasing NaCl concentration and elicitation duration. The results of this study provide an alternative strategy for increasing the antioxidant capacity of cowpea through NaCl-elicitation. Thus, it can be a reference for developing cowpea-based functional food.

1. Introduction

Legumes are potential sources of antioxidant. Meanwhile, germination has been known as a simple and effective strategy to enhance the antioxidant potential of legumes [1][2][3]. Several studies have reported that legumes sprout exhibit a higher antioxidant capacity than the beans [4][5]. Germination with elicitation technique resulted on the improvement in antioxidant capacity of legumes rather than that prepared without elicitation [6][7]. Elicitation is an effective technique to improve antioxidant capacity in plants through the activation of defense system which includes both non-enzymatic and enzymatic antioxidant production as the elicitation stress response [8][9][10]. There are two types of elicitor, namely biotic and abiotic elicitors. The implementation of elicitation using abiotic elicitor is relatively cheaper and easier rather than using biotic elicitor [9]. NaCl is one kind of abiotic elicitor that can be applied to plants [11][12]. Swieca reported that lentil sprouts prepared using NaCl as elicitor produces higher antioxidant capacity than other abiotic elicitors [9]. Antioxidant capacity of common bean and mung bean could be improved by elicitation using NaCl as the elicitor [13][7].
Saha [14] found that the increase of NaCl concentration (50, 100, 150 mM) would increase the antioxidant activity in mung bean sprouts. Meanwhile, NaCl concentration, elicitation duration is another factor that affects germination process. Soaking the seed in NaCl solution will trigger osmotic potential which resulted in inhibition of seed imbibition process. Moreover, it can cause an accumulation of toxic ions such as Na+ and Cl- in the embryo. This accumulation will trigger stress condition in plants thus the balance between ROS production and ROS catabolism will be disturbed. Overproduction of ROS can cause cell death [15].

Compared to other legumes, cowpea is a legume that is easily cultivated and has relatively affordable price. Cowpea (*Vigna unguiculata*) is a multifunctional legume that have higher productivity level than soybean, more tolerant to the environmental stresses, and has the ability to help restore soil fertility [16] [17] [18] [19]. Cowpea also exerted various health benefit, including anti-cancer, anti-diabetic, and anti-hyperlipidemic [17], related to the phenolic antioxidant compounds [20]. Several studies also stated that cowpea is a potential antioxidant source due to its phenolic and flavonoid compounds [21] [22] [23]. Nevertheless, despite of the several advantages of cowpea, it has not been used optimally [24] [25].

Previous studies reported the improvement of antioxidant capacity in the sprouts of lentil [9], mung bean [14], and common bean [13] which prepared using NaCl-elicitation along with an increase in NaCl concentration. Nonetheless, research about the effect of elicitation duration on the improvement of antioxidant capacity of legumes sprouts has not been reported previously. The present research aims to evaluate the effect of various concentrations of NaCl (50, 100, 150 mM) and different elicitation durations (8, 12, 16 hours) on the increase of the antioxidant capacity (total phenolic content, radical scavenging activity and reducing power) and germination power of the cowpea sprouts. The correlation between the total phenolic content and the antioxidant activity of the cowpea sprouts was also analyzed. To the best of our knowledge, study on the enhancement of antioxidant capacity using elicitation technique with different NaCl concentration and elicitation duration in cowpea sprouts has not been carried out. Thus, this study investigates an increase in total phenolic content and antioxidant activity (radical scavenging and reducing power) in cowpea sprouts as a response to NaCl stress.

2. Materials and Methods

2.1. Chemicals

All reagents used in this study were analytical grade. Na₂CO₃, NaH₂PO₄.2H₂O, Na₂HPO₄.2H₂O, K₂Fe(CN)₆, HCl, FeCl₃.6H₂O, Trichloroacetic acid (TCA), Ethanol, and Methanol were purchased from Merck Millipore Co. (Darmstadt, Germany). Meanwhile, Folin-Ciocalteau’s phenol reagent, 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), Gallic Acid, Ascorbic Acid were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Preparation of sprouts

Cowpea (*Vigna unguiculata*) seeds were obtained from the local market in Surakarta, Indonesia. The selected seeds were rinsed three times with distilled water and then soaked in NaCl at different concentrations (0, 50, 100, 150mM) with a ratio of 1:3 w/v and at different elicitation durations (8, 12, 16 hours). The elicited seeds were germinated for 48 hours at room temperature on bamboo weaving (15cm x 15cm). During germination, the seeds were sprayed with distilled water every 12 hours.

2.3. Extract Preparation

The dehulled cowpea sprouts (2 grams) were extracted with 20 ml of 80% methanol at 50°C using a water bath shaker (SWB 20, Fisher Scientific Haake, Germany) at 200 rpm for 2 hours. The mixture was centrifuged using PLC-05 centrifuge, Gemmy, Taiwan, at 10,000 rpm for 15 minutes to obtain a clear supernatant. The supernatant was collected and stored in an amber bottle at 10°C until further analysis.
2.4. Total Phenolic Content Analysis
Total phenolic content was determined based on the method described by Singleton [26]. Total phenolic content was expressed as µM gallic acid equivalent (GAE)/g sample (dry weight, DW).

2.5. Radical Scavenging Activity Test
Radical scavenging activity was analyzed using DPPH free radical method [27]. The free radical scavenging activity was expressed as µM gallic acid equivalent activity (GAEA)/g sample (dry weight, DW).

2.6. Reducing Power Test
The reducing power was measured using the method developed by Berker et al., and expressed as µM ascorbic acid equivalent activity (AAEA)/g sample (dry weight, DW) [28].

2.7. Germination Power
Germination power was measured according to Yang et al., and expressed as percentage [29].

2.8. Statistical Analysis
Data were presented as mean and standard deviations. The data of total phenolic content, radical scavenging as well as the reducing power of cowpea sprouts were analyzed using the IBM SPSS Statistics 22 (SPSS Inc., Chicago, USA) by General Linear Model (Univariate Analysis of Variance). Duncan’s Multiple Range Test (DMRT) was used to evaluate the significant difference between means (p<0.05). Furthermore, Pearson’s correlation analysis was used to determine the correlation between the total phenolic content and DPPH radical scavenging activity as well as reducing power.

3. Results and Discussion
3.1 Effect of Different NaCl Concentrations (50, 100, 150 mM), Elicitation Durations (8, 12, 16 hours) and their Interaction toward Antioxidant Capacity (Total Phenolic Content, radical scavenging activity and reducing power) of Elicited Cowpea Sprouts
Based on statistical analysis, the total phenolic compounds, as well as radical scavenging activity and the reducing power of elicited cowpea sprouts was significantly influenced by NaCl concentration. However, NaCl elicitation duration did not significantly affect the total phenolic compounds of the elicited cowpea sprouts (p>0.05). The interaction between NaCl concentration and elicitation duration had no significant effect on the antioxidant capacity of elicited cowpea sprouts (p>0.05).

Phenolic compound was often induced by abiotic stress for enhancing plant protection [30]. Table 1 shows elicited sprouts using 100 mM and 150 mM NaCl produce higher total phenolic content than those using 50 mM NaCl. This result confirms previous research by Swieca in the total phenolic content of lentil sprouts [9]. ROS accumulation in the plant’s cell due to osmotic pressure disruption and toxic ion from salt during elicitation leads the phenolic overproduction [31]. An increase in total phenolic content in cowpea sprouts prepared with NaCl elicitation was caused by salinity stress that triggers osmotic stress tolerance through osmolyte formation, such as proline. Higher NaCl concentration leads increasing of proline accumulation [32]. Shetty stated that proline synthesis is related with overproduction of erythrose-4-phosphate that plays a role in shikimate and phenylpropanoid pathway which induce phenolic compounds synthesis [33]. On the other hand, the highest total phenolic content which was produced from elicitation with 150 mM NaCl was not significantly different with those elicited by 100 mM NaCl. It might be 100 mM NaCl was the highest concentration that can be applied to enhance total phenolic content in cowpea sprouts. This result was similar to Taibi [13] in which Phaseolus vulgaris elicited by 100 mM NaCl. It had higher total phenolic compound than 50 mM NaCl but it decreased when it was elicited by 200 mM NaCl.

Different concentrations of NaCl also gave significant effect (p<0.05) on radical scavenging and reducing power in elicited cowpea sprouts (Table 1). Cowpea sprouts elicited by 150 mM NaCl had higher radical scavenging activity than those elicited by other concentrations. Several studies also
showed the high correlation between total phenolic content and DPPH radical scavenging, among others 0.637 in mung bean and 0.760 in soybean [5], and 0.966 in buckwheat [34]. Reducing power in cowpea sprouts elicited by different concentrations of NaCl also shows similar result with radical scavenging activity (Table 1). Cowpea sprouts elicited by 150 mM NaCl produces higher reducing power than those elicited by 50 mM and 100 mM NaCl. This result is in line with previous study by Swieca in which lentil sprouts elicited by 300 mM NaCl show higher reducing power than those elicited by 100 mM NaCl [9]. Swieca also stated that reducing power in elicited lentil sprouts is attributed by total phenolic content due to their high correlation (0.91) [9].

Table 1. Effect of NaCl concentration and elicitation duration toward antioxidant capacity (total phenolic content, DPPH radical scavenging and reducing power) of elicited cowpea sprouts.

| Treatment | Parameters | Total Phenolic (µM GAE/g sprouts (db)) | DPPH Radical Scavenging (µM GAE/g sprouts (db)) | Reducing Power (µM AAE/g sprouts (db)) |
|-----------|------------|----------------------------------------|-----------------------------------------------|----------------------------------------|
| NaCl      | 50         | 8373.67±435.29a                        | 811.70±52.78a                                | 6767.21±316.98a                        |
| Concentration (mM) | 100         | 9285.44±355.96b                        | 833.66±51.96a                                | 6867.55±431.22a                        |
| (mM)      | 150        | 9650.08±328.95b                        | 902.74±51.08b                                | 7447.34±354.82b                        |
| Elicitation Duration (hours) | 8          | 8721.86±685.47a                        | 801.32±58.54a                                | 6754.03±416.95a                        |
|           | 12         | 9149.54±541.57a                        | 846.64±37.74a                                | 6892.15±334.53a                        |
|           | 16         | 9437.79±584.67a                        | 900.14±53.07b                                | 7435.91±370.79b                        |

Different superscripts in the same column in similar treatment mean significant differences (p<0.05)

Besides NaCl concentration, elicitation duration also affects the antioxidant capacity of elicited cowpea sprouts. In the present study, although elicitation duration did not affect the total phenolic content (p>0.05), yet the highest elicitation duration (i.e., 16 hours) produced significantly higher than radical scavenging activity as well as reducing power than those elicited for 8 and 12 hours. This result may indicate that the antioxidant activity is affected by other antioxidant compounds besides phenolic as a response to the NaCl stress. Accumulation of toxic ions due to NaCl as an elicitor leads to ionic and osmotic stresses as well as water deficit [35]. Accordingly, the plants activate a series of defense system as a response to stress [8], including by increasing enzymatic and non-enzymatic antioxidant production [9]. For instance, phenolic acid, flavonoid, and tannin are naturally synthesized to protect plants from stress during the growth [4]. The antioxidant compounds such as phenolic and flavonoids have a high correlation to the antioxidant activities [5] [9] [36]. Based on this research results, the correlation between radical scavenging activity and the reducing power of elicited cowpea sprouts was also determined. Data is presented in Table 2.

Table 2. Correlation coefficient between total phenolic and antioxidant activity of elicited cowpea sprouts.

| Antioxidant activity       | Correlation coefficient (r) |
|---------------------------|----------------------------|
| DPPH radical scavenging   | 0.805**                    |
| Reducing power            | 0.785**                    |

**Correlation analysis using Pearson correlation; p<0.01

3.2. Correlation between Total Phenolic Content and Antioxidant Activity (DPPH radical scavenging and reducing power) of Elicited Cowpea Sprouts

The correlation between total phenolic content and antioxidant activity (DPPH radical scavenging and reducing power) are presented in Table 2. Based on Table 2, total phenolic content had a very high correlation with DPPH radical scavenging (r=0.805, p<0.01) and reducing power (r=0.785, p<0.01). R value from Table 2 indicated that total phenolic content had higher correlation with radical scavenging activity rather than with reducing power. This result is similar with previous studies on cowpea [23], mung bean and soybean sprouts [5]. It might be caused by sinapic acid, a common
phenolic compound in plants, which has been reported to play an important role on radical scavenging by transfer of the labile H atoms to radical compounds [4]. In addition, there are several phenolic compounds that also have a role on radical scavenging activity and reducing power, such as gallic acid, p-coumaric acid, neochlorogenic acid, caffeic acid, ferulic acid, cinnamic acid, ellagic acid, and benzoic acid [4] [23] [37].

Based on the findings, it can be stated that as an elicitor, NaCl affects the antioxidant capacity of elicited cowpea sprouts. Nevertheless, it also affects the plant growth. Saha et al. stated that an increase in NaCl concentration could inhibit the growth of mung bean sprouts. Therefore, research about the effect of elicitation on cowpea germination power is needed [14].

3.3. Effect of Different NaCl Concentrations (50, 100, 150 mM), Elicitation Durations (8, 12, 16 hours) and their Interaction toward Germination Power of Elicited Cowpea Sprouts

The statistical analysis carried out in the present study exhibited that the germination power of the elicited cowpea decreased along with the increase in NaCl concentration and elicitation duration. This result is in line with previous studies investigating lentil sprouts [38] and mung bean sprouts [39] which showed the higher the NaCl concentration, the lower the germination power. Likewise, high NaCl concentration will decrease osmotic potential and eventually inhibit water uptake by seed [39] [40] [41]. A lower cowpea sprouts germination power was observed in a higher elicitation duration. The higher NaCl accumulation and higher elicitation duration induce higher stresses [14] [35], which further impact on the decline in germination power.

| NaCl Concentration (mM) | Germination Power (%) | Elicitation Duration (hours) |
|------------------------|-----------------------|------------------------------|
|                        | 8                     | 12                           | 16                           |
| 50                     | 83.40±0.88g           | 81.45±1.59g                  | 76.49±1.25f                  |
| 100                    | 69.63±0.44e           | 68.59±1.61e                  | 59.25±1.39d                  |
| 150                    | 53.79±1.62c           | 36.28±1.64b                  | 17.21±1.04a                  |

Different superscripts mean significant differences (p<0.05)

The statistical analysis also indicated that the interaction between NaCl concentration and elicitation duration had significant effect (p<0.05) on the germination power of elicited cowpea sprouts. Table 3 presents the germination power of elicited cowpea sprouts prepared using various NaCl concentration for 8, 12, and 16 hours elicitation duration. Two elicitation conditions exhibit the highest germination power, namely elicitation by 50 mM NaCl for 8 hours and by 50 mM NaCl for 12 hours. This result also indicates that elicitation using 50 mM NaCl for 8 or 12 hours obtain similar NaCl accumulation level, thus producing similar germination inhibition level.

4. Conclusion
In the present study, elicitation by using NaCl has been investigated in which the findings prove that this technique affects the antioxidant capacity and germination power of cowpea sprouts. Elicitation using the higher NaCl concentration produces the higher antioxidant capacity (total phenolic content, radical scavenging activity and reducing power of elicited cowpea sprouts). Besides the concentration of NaCl, elicitation duration also affects the antioxidant activity of cowpea sprouts, including radical scavenging activity and reducing power. This study also shows the significant correlation (p<0.01) between total phenolic content and antioxidant activity (radical scavenging activity and reducing power) of elicited cowpea sprouts. But, higher NaCl concentration and longer elicitation duration potentially inhibit the germination process as indicated by lower germination power. Thus, the
elicitation technique using 50 mM NaCl for 8 hours can be an effective strategy to develop cowpea-based functional food due to its high antioxidant capacity and germination power.

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