Elicitation under salinity stress increases flavonoid content and antioxidant activity in cowpea (*Vigna unguiculata*) sprouts

F M Rajendra, L S Kristiani and S Ariviani

Department of Food Science and Technology, Faculty of Agriculture, Universitas Sebelas Maret, Jl. Ir. Sutami 36 A, Kentingan, Surakarta 57126, Indonesia

Email: setya_ariviani@yahoo.com; setyaningrum_ariviani@staff.uns.ac.id

**Abstract.** Legumes are often used as a source of natural antioxidant. Elicitation is a promising alternative way of improving antioxidant compounds in legumes sprouts, such as flavonoid compound. NaCl stress can be used as one of abiotic elicitation that induced non-enzymatic defense in a plant, thus increases secondary metabolites which enhance the antioxidant capacity. However, its effects on cowpea (*Vigna unguiculata*) germination need to be more studied. In this study, we germinated cowpea under increasing salinity (0, 50, 100, 150mM NaCl) to investigate its effect on the total flavonoid content and antioxidant activity (radical scavenging activity and reducing power). Total flavonoid content and radical scavenging activity of cowpea sprouts increase along with increasing NaCl concentration. Meanwhile, only 150mM NaCl showed significantly higher reducing power among other concentrations. Total flavonoid content have a high correlation with radical scavenging activity (r=0.962; p<0.01) but not correlated with reducing power (r=0.137; p>0.05). This research results proved that elicitation using 150mM NaCl could be used as one of the strategies to enhance bioactive compound and antioxidant activity in legumes, thus increasing its potential to be developed as an antioxidant-based functional food.

1. Introduction

Reactive Oxygen Species (ROS) is one type of free radical compound [1]. In a normal cell, there is a balance condition between ROS production and antioxidant [2]. Excessive ROS production in the human body can cause oxidative stress which leads to oxidative damage towards fat, protein, and DNA [3,4]. Therefore, the intake of rich antioxidant food is needed to keep the balance of ROS and antioxidant.

Legume can be used as one of the natural antioxidant sources. Legume contains several substances that can improve health, such as phenolic and flavonoid. Several studies have proved that cowpea (*Vigna unguiculata*) contains phenolic and flavonoid compounds [5,6].

Germination process is a common way to increase antioxidant capacity in legume [7–9]. There are several studies that report the enhancement of antioxidant capacity in cowpea during the germination process [10,11]. Elicitation during germination process is one of the factors which affect the increase of antioxidant compound in legume sprouts.

Elicitation is an efficient technique to increase phenolic and other antioxidant compounds of plants as the stress response [12,13]. Elicitation can be applied using both of biotic and abiotic elicitor. The
advantages of using abiotic elicitor are cheaper and relatively easy to be applied [12]. Salt stress is one of abiotic elicitation that can be applied to plants [14,15]. Na\(^+\) ion gives toxicity to the plant, while Cl\(^-\) is anion which most frequently found in soil or water which has high salt content. Na\(^+\) and Cl\(^-\) ions are easier to get inside a cell compared to the other ions. An excess of Na and Cl ions induce plant’s cell damage. Moreover, the high NaCl concentration outside the plant’s cell caused stress due to water deficit as a result of osmotic pressure [16]. Swiec a [12] reported that elicitation using NaCl in lentil sprouts produced higher antiradical activity and reducing power rather than other abiotic elicitation.

Previous studies reported improvement of the antioxidant capacity of legumes sprouts elicited with NaCl along with increasing of NaCl concentration, such as lentil [12], mungbean [17,18], and common bean [19]. To the best of our knowledge, the study on the antioxidant capacity enhancement using NaCl elicitation with a various concentration in cowpea sprouts hasn’t been reported. Thus we hypothesized an increase in flavonoid levels and antioxidant activity (radical scavenging and reducing power) are going to be observed in cowpea sprouts as a response to NaCl stress. Therefore, the present research aims to evaluate the effect of increasing NaCl levels (0, 50, 100, 150mM) on the improvements of total flavonoid content as well as antioxidant activity (radical scavenging activity and reducing power) of cowpea sprouts. The correlation between the total flavonoid content and the antioxidant activity was also analyzed.

2. Methods

2.1 Preparation of Sprouts
Cowpea (Vigna unguiculata) seeds were purchased from the local market in Surakarta, Indonesia. Selected seeds (not wrinkled and not hollow seeds) were rinsed thrice with distilled water. Afterward, the seeds were soaked in NaCl solution (0, 50, 100, 150mM) with a ratio of 1:3 w/v for 8 hours at room temperature. The elicited seeds were germinated for 48 hours on bamboo weaved (15 cm x 15 cm). During germination, seeds were sprayed with distilled water every 12 hours.

2.2 Extract Preparation
Dehulled cowpea sprouts (2 grams) were extracted with 20 ml of the methanol: water (80:20, v/v) at 50°C using a water bath shaker (SWB 20, Fisher Scientific Haake, Germany) at 200 rpm for 2 hours. The extract was separated by centrifugation (PLC-05 Centrifuge, Gemmy, Taiwan) at 10,000 rpm for 15 minutes. The clear supernatant was collected and stored in an amber bottle at 10°C until further analyzed.

2.3 Total Flavonoid Content Analysis
Total flavonoid content was determined following the method described by Pękal and Pyrzynska [19]. Total flavonoid content was expressed as QE (µM Quercetin Equivalent/g sample dry weight).

2.4 Radical Scavenging Activity Test
Radical scavenging activity was analyzed using ABTS\(^+\) free radical method [20]. The free radical scavenging activity was expressed as TEAC (Trolox Equivalent Antioxidant Capacity/g sample dry weight).

2.5 Reducing Power Test
Determination of reducing power was conducted using the method described by Berker et al [21]. The reducing power was expressed as AAEA (µM Ascorbic Acid Equivalent Activity/g sample dry weight).

2.6 Statistical Analysis
Data were presented as mean and standard deviations. The data of total flavonoid content, radical scavenging as well as the reducing power of cowpea sprouts were analyzed using the program IBM
SPSS Statistics 22 (SPSS Inc., Chicago, USA) by analysis of variance (ANOVA). Duncan’s multiple range test (DMRT) was used to evaluate the significant differences between mean (p<0.05). Pearson correlation analysis was used to determine the correlation between total flavonoid content and antioxidant activity.

3. Results and Discussion
Salt stress increases ROS accumulation due to the increase of the respiration rates as a response to the stress [22]. Plants will develop a defense mechanism to reduce ROS excess, through secondary metabolite production such as phenolic and flavonoid compounds [12]. Total flavonoid content and radical scavenging activity, as well as the reducing power of cowpea sprouts which prepared with elicitation using various NaCl concentrations, were presented in Table 1.

| NaCl concentration (mM) | Total flavonoid content (µM QE/g sprout dry weight) | ABTS•⁺ radical scavenging activity (mM TEAC/g sprout dry weight) | Reducing power (µM AAEA/g sprout dry weight) |
|-------------------------|-----------------------------------------------------|---------------------------------------------------------------|---------------------------------------------|
| 0                       | 250.41 ± 2.42ᵃ                                       | 952.16 ± 26.47ᵃ                                                | 6790.20 ± 43.02ᵃ                             |
| 50                      | 398.49 ± 8.72ᵇ                                       | 1130.11 ± 43.01ᵇ                                               | 6484.93 ± 226.25ᵃ                             |
| 100                     | 418.01 ± 7.19ᶜ                                       | 1192.89 ± 41.54ᶜ                                               | 6539.56 ± 188.21ᵃ                             |
| 150                     | 444.98 ± 8.71ᵈ                                       | 1259.51 ± 29.72ᵈ                                               | 7237.60 ± 283.53ᵇ                             |

Different superscript within similar column means significant differences (p<0.05)

NaCl concentration significantly affects the total flavonoid content of cowpea sprouts. The flavonoid content increased as the NaCl concentration raised (Table 1). Taib et al [18] that studied the effect of salt stress on antioxidant defense systems in Phaseolus vulgaris reported a similar result. Total flavonoid content in Phaseolus vulgaris sprouts increase along with increasing NaCl concentration. Study of Świeca [12] showed that lentil sprouts elicited with higher NaCl concentration showed significantly higher (p<0.05) total flavonoid content.

The results in Table 1 also exhibited that the total flavonoid content of cowpea sprouts with NaCl elicitation was significantly higher than that of cowpea sprouts without elicitation (0mM NaCl). ROS accumulation in the plant’s cell due to osmotic pressure disruption and toxic ion from salt during elicitation lead the phenolic and flavonoid overproduction [22]. Increasing of total flavonoid 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 to increasing of proline accumulation [23]. Shetty [24] 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. Moreover, Świeca [12] reported that increasing of NaCl concentration cause enhancement of phenolic compound in lentil sprouts. One of the major phenolic compounds in cowpea sprout is p-coumaric acids [10, 25,26]. P-coumaric acids might be involved in flavonoids biosynthesis via Coumaryl Coa [10,27–29].

Flavonoid and phenolic compounds exhibit several health benefits related to the antioxidant capacity [30]. Based on its mechanism, an antioxidant is divided into two types, those are a primary and secondary antioxidant. The primary antioxidant has the ability to be free radical scavenger by electron or hydrogen donating, while secondary antioxidant has the ability to reduce another component in order to inhibit free radical generation indirectly [31].

NaCl concentrations not only affects total flavonoid content but also significantly (p<0.05) affects the radical scavenging activity of cowpea sprouts. The higher NaCl concentration showed significantly higher (p<0.05) radical scavenging activity. This result in agreement with Świeca [12].
who reported that lentil sprouts with elicitation using 300mM exhibited higher radical scavenging activity than that of 100mM NaCl (p<0.05). The radical scavenging activity enhancement observed in this study could be attributed to the increase in total flavonoid content.

Besides radical scavenging activity, increasing NaCl concentration as elicitor also significantly (p<0.05) affects the reducing power of cowpea sprouts. It was interesting that cowpea sprouts without elicitation exhibited the reducing power that did not significantly different (p>0.05) from those elicited using 50 and 100mM NaCl, although it showed significantly lower total flavonoid content. This phenomenon is possible because of the reducing power did not only attributed to the flavonoid compounds but also other reductants such as ascorbic acid. Guo et al. [32] reported that the increase in NaCl concentrations decreased ascorbic acid content in the sprouts of three broccoli cultivars. It was could be explained by two mechanisms, i.e NaCl stress inhibits the activity of ascorbic acid generating enzyme as well as ascorbic acid utilization to protect osmotic stress caused by salinity. Reduction of ascorbic acid level in cowpea sprouts elicited using various NaCl concentrations need further investigations.

### Table 2. Correlation coefficient between flavonoid total and antioxidant activity of NaCl elicited cowpea sprout.

| Antioxidant activity          | Correlation coefficient (r) |
|------------------------------|-----------------------------|
| ABTS•⁺ radical scavenging    | 0.962**                     |
| Reducing power               | 0.137                       |

** Correlation analysis using Pearson correlation; p<0.01

Table 2 showed that the total flavonoid content was significantly correlated (r=0.962, p<0.01) with radical scavenging activity but not correlated with reducing power (p>0.05). It might be caused by major flavonoid contents in cowpea, such as flavonols and flavan-3-ols which have OH group in C3 [33,34]. OH group location in flavonoid compounds will affect energy needed to donate an electron. Flavonols show higher radical scavenging activity due to low energy needed to donate an electron. Moreover, flavan-3-ols also have high radical scavenging activity due to the effect of OH group in C3 and strengthened by the presence of 3’-4’-catechol [35,36]. Several studies showed that reducing power might be more attributed by other antioxidant compounds, such as ascorbic acid than phenolic and flavonoid compound [37,38].

### 4. Conclusions

The result of this study showed that increasing of the total flavonoid content, radical scavenging activity, and reducing power are affected by NaCl concentration used in elicitation. The higher NaCl concentration produces cowpea sprouts with higher antioxidant capacity. Although the radical scavenging activity of cowpea sprouts has a significantly high correlation to the total flavonoid content, the reducing power did not correlate to the total flavonoid content. The reducing power of cowpea sprouts may be more attributed by other reducing agents such as ascorbic acid rather than flavonoid compounds. Elicitation using 150mM NaCl is a promising strategy to increase the antioxidant capacity of cowpea sprouts thus more potential to be developed as an antioxidant-based functional food.

### References

[1] Gulcin I 2012 *Arch Toxicol.* **86** 345–91.
[2] Birben E, Sahiner U M, Sackesen C, Erzurum S and Kalayci O 2012 *World Allergy Organ J.* **5** 9–19.
[3] Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S and Dhama K 2014 *Biomed Res Int.* **2014** 1–19.
Sies H 2015 *Redox Biol.* **4** 180–3.

Cai R, Hettiarachchy N S and Jalaluddin M 2003 *J Agric Food Chem.* **51** 1623–7.

Ojwang L O, Dykes L and Awika J M 2012 *J Agric Food Chem.* **60** 3735–44.

Tang D, Dong Y, Ren H, Li L and He C 2014 *Chem Cent J.* **8** 1–9.

Tarzi B G, Gharachorloo M, Baharinia M and Mortazavi A 2012 *Iran J Pharm Res.* **11** 1137–43.

Saleh H M, Hassan A A, Mansour E H, Fahmy H A and El-Bedawey A E F A 2018 *J Saudi Soc Agric Sci.* **2017** 1–8.

Khang D, Dung T, Elzaawely A and Xuan T 2016 *Foods.* **5** 27–36.

Aguilera Y, Díaz M F, Jiménez T, Benítez V, Herrera T, Cuadrado C, Martín-Pedrosa M and Martín-Cabrejas M A 2013 *J Agric Food Chem.* **61** 8120–5.

Świeca M 2015 *Saudi J Biol Sci.* **22** 409–16.

Dziki D, Gawlik-Dziki U, Kordowska-Wiater M and Domań-Pytka A 2015 *J Chem.* **2015** 1–8.

Ramakrishna A and Ravishankar G A 2011 *Plant Signal Behav.* **6** 1720–31.

Gorelick J and Nirit B 2014 *Adv Agron.* **124** 201–30.

Azooz M M and Ahmad P 2015 *Legumes under Environmental Stress: Yield, Improvement and Adaptations* (Chichester: Wiley Blackwell).

Saha P, Chatterjee P and Biswas A K 2010 *Indian J Exp Biol.* **48** 593–600.

Taibi K, Taibi F, Ait Abderrahim L, Ennajah A, Belkhodja M and Mulet J M 2016 *South African J Bot.* **105** 306–12.

Pękal A and Pyrzynska K 2014 *Food Anal Methods.* **7** 1776–82.

Re R, Pellegrini N, Proteg gente A, Pannala A, Yang M and Rice-Evans C 1999 *Free Radic Biol Med.* **26** 1231–7.

Berker K I, Güçlü K, Tor I and Apak R. 2007 *Talanta.* **72** 1157–65.

Abogadallah G M 2010 *Plant Signal Behav.* **5** 369–74.

Thakur M and Sharma A D 2005 *J Arid Environ.* **62** 517–23.

Shetty K 2004 *Process Biochem.* **39** 789–803.

Gutiérrez-Uribe J A, Romo-Lopez I and Serna-Saldívar S O 2011 *J Funct Foods.* **3** 290–7.

Singh B, Singh J P, Kaur A and Singh N 2017 *Food Res Int.* **101** 1–16.

Gan R, Lui W, Wang M and Sui Z 2016 *Functional Foods in Health and Diseases* 6 519–35.

Chemler J A, Yan Y and Koffas M A G 2006 *BioMed Central.* **9** 1–9.

Ngameni B 2013 *Medical Plant Research in Africa* (New York: Elsevier).

Chen J, Lu J, Zhao H, Fan W, Dong J, Kong W, Sun J, Cao Y and Cai G 2008 *J Agric Food Chem.* **55** 10994–1001.

Lobo V, Patil A, Phatak A and Chandra N 2010 *Pharmacogn Rev.* **4** 118–26.

Guo L, Yang R, Wang Z, Guo Q and Gu Z 2014 *Int J Food Sci Nutr.* **65** 476–81.

Apea-Bah F B, Minnaar A, Bester M J and Duodu K G 2014 *Food Chem.* **157** 157–66.

Nassourou M A, Njitang Y N, Noubissiie T J B, Nguimbou R M and Bell J M 2016 *Crop J.* **4** 391–7.

Fidrianny I, Elviana D and Ruslan K 2016 *Int J Pharmcogn Phytochem Res.* **8** 470–6.

Heim K E, Tagliaferro A R and Bobilya D J 2002 *J Nutr Biochem.* **13** 572–84.

Chai M Y, Azlan A, Al-Sheraji S H, Hassan F A and Nagendra Prasad K 2014 *Pakistan J Nutr.* **12** 1036–41.

Ramesh C K, Rehman A, Prabhakar B T, Vijay Avin B R and Aditya Rao S J 2011 *J Appl Pharm Sci.* **1** 99–103.