# Profile of Protein Levels Some Tobacco Varieties (Nicotiana tabacum L.) On Waterlogging Stress

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# Profile of Protein Levels Some Tobacco Varieties (*Nicotiana tabacum* L.) On Waterlogging Stress

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**ABSTRACT**

Tobacco is a high-value crops that are sensitive to waterlogging stress. Some tobacco varieties have been widely cultivated in Indonesia, including Jepon Mawar, Jepon Banyak and Rejeb. Some species have different abilities to withstand the conditions of waterlogging stress by morphological adaptation, anatomy, physiology, and metabolic pathway changes. Changes in protein profiles is one form of plant defense response to waterlogging stress. Profile proteins experienced upregulation in hypoxia and anoxia conditions when gripped waterlogging stress known as anaerobic polypeptides. In addition to the waterlogging stress will cause the decrease in chlorophyll levels as a result of chlorosis during the stress. The purpose of this research is to know protein profile and chlorophyll content of several varieties of tobacco (Nicotiana tabacum L.): Rejeb, Jepon Mawar and Jepon Many against stagnant waterlogging stress. The protein profile was analyzed by SDS-PAGE method. While the chlorophyll content was analyzed by spectrophotometric method. The protein profiles expressed from the three test varieties were present in the molecular weight range 85.38-153.33 kDa. Proteins with a molecular weight of 85.38 kDa are thought to have similarities to the Peroxsidase group (BM = 85 kDa) that play a role in the ROS detoxification process. While the chlorophyll content in the three varieties decreased with the increasing of waterlogging stress except on Rejeb varieties treated by 175% and Jepon Mawar varieties on 200% waterlogging stress treatment.



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## 1. INTRODUCTION

Waterlogging of an abiotic stresses which may affect growth and yields. One of the causes of waterlogging is the high rain insensity. The main cause of damage to the plant during the waterlogging is the low oxygen, which indicated the existence of wilting symptoms due to the absorption of nutrients and water that hampered [1]. Waterlogging can also cause the condition of hypoxia and anoxia in the soil. Stress reduces the pool of gas exchange between the plant tissue and air as gas diffusion layer in water 10,000 times slower than in air [4]. Despite the low oxygen availability is an important factor affecting plant growth, soil chemical element content, such as pH and redox potential, is also changing under stress conditions inundation and can also affect the survival and growth of plants [5,6

Lack of oxygen stimulates anaerobic fermentation that affect less well on some morphological and physiological processes, such as photosynthesis, energy metabolism, redox potential, gene expression, as well as

degradation and protein synthesis [7]. One form of plant responses to abiotic stress is the change in protein expression and post-translational modification of proteins to activate the defense system in the face of stress [8]. The protein profile changes in hypoxia. In addition, also expressed specific proteins that have upregulation in this case is anaerobic polypeptides. The enzyme plays a role in the metabolism of glucose, glycolysis, fermentation [9], hormone synthesis, programmed cell death [10].

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Response of the other plant in response to the stress puddle is a decrease in the conductance of stomata (Folzer et al., 2006), changes in hormone balance (Else et al., 2001), decreased transpiration and inhibition of photosynthesis rate due to a decrease in leaf chlorophyll content (Cao and Conner, 1999). These responses occur within hours or days, depending on the level of tolerance of plant species (Striker et al., 2005). The decline of chlorophyll and chlorophyll degradation massively occur during leaf senescence, fruit ripening, and also in response to environmental stress (Hortensteiner and Bernhard, 2010). The decline in the chlorophyll content due to intake of nutrients, especially of N low due to damage to the root system due to flooding.

Determination of protein profiles and chlorophyll content of some varieties of tobacco in response to stress puddle provide a better understanding of its function in adaptation to stress.

## 2. RESEARCH METHOD

### 2.1 Preparation of tobacco seeds

Tobacco varieties seed in this study include var. Srumpung, Dixie Bright and Somporis. The seeds were germinated in germination media containing compost and chaff (2:1). Germination was carried out for 15 days after seeding (15 das). Germinated seeds were then pricked (until seedlings aged 54 das) and transferred *pottray*.

### 2.2. Planting tobacco seeds in polybag

54 das tobacco seedlings were transferred to non-perforated polybags. Planting medium used in this procedure was compost and chaff with a ratio of 4:1. Each polybag contains 1 kg of planting medium. 5 gr NPK fertilizer was applied for each plant. Pest and disease control was done using insecticides organtrin 1.5 ml / L. The control carried out at the time of old tobacco plants (15 HST). Tobacco plants were grown for 3 weeks (21 days after planting (dap).

### 2.3. Field capacity measurement

Measurements of field capacity were conducted to determine the volume of the water in the treatment of *waterlogging stress*. Planting media that were placed in polybag *were watered* until the water passed through the media. The media was subsequently *left to stand for* 3 days until there were no *the dripping water occurred*. Furthermore, the media were directly weighted. Meanwhile, dry weight media were measured by placing the

planting medium in the oven at 100° C for 24 hours until reaching a constant weight. Field capacity is calculated using the formula:

$$W = \frac{Tb - Tk}{Tk} X\ 100\ \%$$

Information:
W : Field Capacity
Tb : Wed Weight (Gram)
Tk : Dry Weight (Gram)

**2.4. Stress Waterlogging Treatment**

*Waterlogging stress* treatments were carried out using 21 dap tobacco plants. *Waterlogging stress* treatment used in this study were 100%, 150%, 175% and 200%. These treatments were applied during 10 days. The volume of water was maintained for 10 days of treatment.

**2.5 Sample Preparation (Protein Extraction)**

Protein profile analysis using protein electrophoresis method. Plants that have been taken are washed with distilled water. A total of 0.250 grams of leaf organs were washed with phosphate buffered saline (PBS) pH 7.4. The organs were homogenized with cold mortar and added 500 µl of protein extract buffer. Homogenate is inserted in a 1.5 ml tube and centrifuged at 10000 rpm at 4 ° C for 10 minutes. The pellet is removed and the supernatant is inserted a new 1.5 ml tube then stored at -20 ºC. For electrophoresis used 12.5% separating gel and 5% stacking gel.

**2.7 Parameters of observation**

Parameters include the observation of profile protein.

**2.8 Data Analysis**

The data chlorophyll content obtained were analyzed using ANOVA Two Way followed by *Tukey* test and descriptive to analysis provile protein.

**3. RESULTS AND ANALYSIS**

**3.1 Protein Profile Jepon Variety**

Based on the results of SDS-PAGE analysis Jepon Many varieties are shown in Figure 1 shows that a protein with a molecular weight of 153.33 kDa decreased expression represented by the thickness of the protein band with increasing level of flooding. Protein band with a molecular weight of 119.51 and 103.98 kDa expressed only in the treatment of 100% waterlogging stress and degradation as indicated by the loss of protein bands on the treatment of 150%, 175% and 200%. The protein band with a molecular weight of 90.48 kDa is expressed only in the concentration of 175% and 200% waterlogging. While the protein with a molecular weight of 85.38 kDa was uniformly expressed on all the treatment of waterlogging stress. The results show that there are some proteins that degrade or decrease expression when the waterlogging stress. In addition there are also proteins that are only expressed at high waterlogging rates such as proteins with molecular weight of 90.48 kDa. The weight of expressed protein molecules differs across all treatments ranging from 85.38-153.33 kDa.

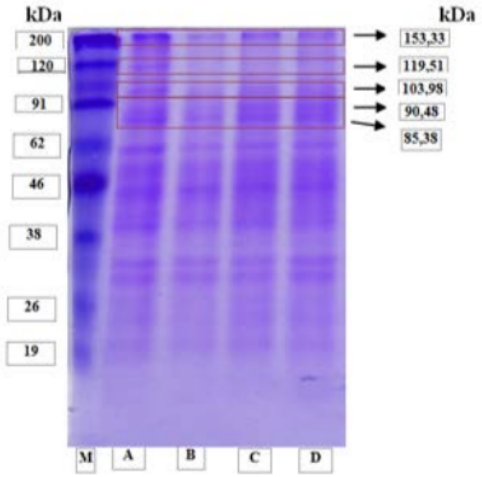


**Figure 1**. Results of Analysis of SDS-PAGE Profiles of Many Jepon Variety Proteins. : (M) Marker; (B) 100% Waterlogging stress above the field capacity; (C) 150% Waterlogging stress above the field capacity (D) 175% Waterlogging stress above the field capacity (E) 200% Waterlogging stress above the field capacity.

Research on tomato plants (Solanum lycopersicum) treated with waterlogging for 14 days showed that expressed proteins ranged in molecular weight 50-110 kDa [21]. The 50-55 kDa molecular weight protein is a member of the Rubisco Large-Subunit (RLS). The 60-61 kDa molecular weight protein is Rubisco Binding Protein (RBP), a molecular weight protein of 93-95 kDa which is an ATP-dependent protease belonging to serine protease (Clp-P), and a protein with a molecular weight of 110 kDa is a protein Rubisco Activase (RA). Based on the results of identification, these proteins play a role in the process of photosynthesis. Rubisco's expression decreased in total dissolved protein during response to the inundation stress. Rubisco has two functions that act as carboxylases that mediate CO2 assimilation and as oxygenase in catalyzing the early photorespiration stage [22]. In addition, the presence of inundation can also induce the formation of ROS (Reactive Oxygen Species) which may lead to the degradation of Rubisco subunit and Rubisco Activase.

### 3.2 Protein Profile Rejeb Variety

In Rejeb varieties, occurring protein with molecular weight of 153.33 kDa increased expression in the treatment of waterlogging stress 175% as indicated by the band thicker than other treatments. Protein with a molecular weight of 119.21 and 103.98 kDa was also expressed on the previous varieties. These proteins are expressed in the treatment of 100% and have been degraded in the treatment of 150%, 175%, 200%. In addition, there is an increased expression of the protein bands with molecular weight of 85.38 kDa when the waterlogging stress treatment 175%, it is seen from the thickness of the protein bands. Band thickness decreases as 200% inundation concentration as shown in Figure 2.

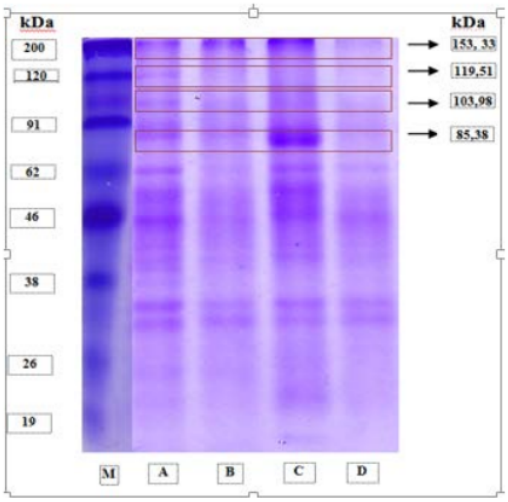


**Figure 2**. Results of Analysis of SDS-PAGE Profiles of Many Jepon Variety Proteins. : (M) Marker; (B) 100% Waterlogging stress above the field capacity; (C) 150% Waterlogging stress above the field capacity (D) 175% Waterlogging stress above the field capacity (E) 200% Waterlogging stress above the field capacity.

According to [23], maize (Zea mays) were flooded for 52 hours showed increased expression of a protein with a molecular weight of 85 kDa and 133 kDa. Protein with a molecular weight of 133 kDa initially decreased expression during the initial 4 hours of waterlogging stress, and increased after 28 to 52 hours of waterlogging stress. While the protein with a molecular weight of 85 kDa significantly increased its expression during 52 hours of waterlogging stress. Based on the results of identification, both proteins are included in the peroxidase group that play a role in the physiological functions of plants including plant development, oxidative stress and processes associated with plant cell walls. Peroxsidase also plays a role in the production and detoxification of ROS (Reactive Oxygen species) caused by stress.

Oxidative stress occurs when unbalanced production and neutralization of ROS within cells. High light, heat, pathogen attack, low oxygen levels, and re-aeration after hypoxia phase can increase ROS production [25]. ROS is produced from molecular oxygen through several reduction steps. Anion superoxide ($O_2$-), hydroxyl radical (OH), singlet oxygen ($^1O_2$) results from the reduction of one or three electrons from oxygen by the reduction energy provided by the electron carriers in mitochondria and chloroplasts [26]. ROS is highly reactive and can cause damage to lipid membranes and proteins [23]. Plants have a protective system to protect mitochondria from overproduction of ROS such as antioxidants (glutathione, ascorbic acid, tocopherol, tannins, ubiquinol, and phenolic acid), ROS scavenging enzymes such as superoxide dismustase (SOD), catalase (CAT), ascorbate peroxidase (APX ), and gluthatione peroxidase (GPX). So it can be predicted that an increase in protein expression with a molecular weight of 85.38 kDa in the Rejeb variety (175% stroke) is one form of protection mechanism of oxidative stress that can damage the lipid membrane and other proteins. It is also supported with Rejeb Reef morphology data of 175% treatment which showed better growth compared to 100% treatment.

### 3.3 Protein Profile Jepon Mawar Variety

In Jepon varieties of roses, also express proteins that appeared in previous varieties as shown in Figure 3, which is a protein with a molecular weight of 119.51 and 103.98 kDa that appears only in the treatment of stress puddle 100%. Protein with a molecular weight of 153.33 kDa stress treatment increased expression as a pool of 200%. Increased expression also occurs in Rejeb varieties with 175% stress treatment. While the protein with a molecular weight of 85.38 kDa expressed only in the treatment of a pool of 100% stress and degradation or decreased expression with increasing level of flooding. So it can be said that the majority of proteins that appear on the treatment of 100% have been degraded in the other treatment.

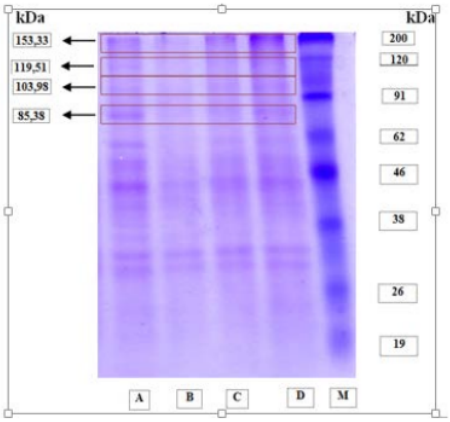


**Figure 3**. Results of Analysis of SDS-PAGE Profiles of Many Jepon Variety Proteins. : (M) Marker; (B) 100% Waterlogging stress above the field capacity; (C) 150% Waterlogging stress above the field capacity (D) 175% Waterlogging stress above the field capacity (E) 200% Waterlogging stress above the field capacity.

## 4. CONCLUSION

Stress can alter protein expression inundation of some varieties of tobacco. SDS-PAGE analysis of tobacco leaf protein indicates that there are 5 different proteins expressed in each variety and treatment of waterlogging stress. proteins are expressed differently in all three test varieties contained in the molecular weight range from 85.38 to 153.33 kDa. Varieties Rejeb increase protein expression at a molecular weight of 85.38 kDa in the treatment of 175%, as indicated by the thickness of the protein bands. The protein is degraded in the varieties of roses Jepon treatment 150%, 175% and 200% and expressed the same in all treatment Jepon Many varieties. Protein with a molecular weight of 85.38 kDa allegedly has in common with Peroxsidase group (molecular weight 85 kDa), which plays a role in detoxification of ROS.

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## REFERENCES

[1] Sairam, R.K., Kumutha, D., Ezhilmathi, K., Deshmukh P.S, dan Srivastava, G.C. "Physiology and biochemistry of waterlogging tolerance in plants". Journal Bio Plant (2008) 52:401–412.
[2] Barrett-Lennard, E.G. "The interaction between waterlogging and salinity in higher plants causes consequences and implications". Journal Plant and Soil (2003) 253:35-44.
[3] Shimmamura, S., Yoshida, S., dan Mochizuki, T. "Cortical aerenchyma formation in hypocotil and advemtitious roots of *Luffa cylindrical* subjected to soil flooding". Journal of Annals Botany (2007) 100:1431–1439.
[4] Armstrong, J. dan Armstrong, W. "Rice: sulphide–induced barriers to root radial oxygen loss, $Fe^{2+}$ and water uptake, and lateral root emergence". Journal Annals of Botany (2005) 96:625–638.
[5] Jackson, M.B., dan Colmer, T.D. "Response and adaptation by plants to flooding stress". Ann. Bot. (2005) **96**: 501-505.
[6] Jackson, M.B. "The impact of flooding stress on plants and crops". School of Biological Sciences, University of Bristol, Bristol. (2003)pp. 1-15.
[7] Jackson, M.B., dan P.C. Ram. "Physiological and molecular basis susceptibility and tolerance of rice plants to comolete submergence". Journal Annals of Botany (2003) 91:227-241.
[8] Hashiguchi, A., Ahsan, N., dan Komatsu, S. "Proteomics application of crops in the context of climatic changes". Food Res. Int. (2010) 43: 1803-1813.
[9] Huang, S., Greenway, H., Colmer, T.D., dan Millar, A.H. "Protein synthesis by rice coleoptiles during prolonged anoxia: implications for glycolysis, growth and energy utilization". Journal Annals of Botany (2005) 96:661-668.
[10]Ahmad, Parvaiz dan Salema Rasool, *Emerging Technologies and Management of Crop Stress Tolerance*. Sandiego: Elsevier 2014.
[11]Ahsan, N., Lee, D.G., Lee, S.H, Lee, K.W., Bahk, J.D., dan Lee, B.H. "A proteomic screen and identification of waterlogging-regulated proteins in tomato roots". Journal Plant and Soil (2007) 295:37-51.
[12]Sach, M.M., Freeling, M., dan Okimoto, R. "The anaerobic proteins of maize". Cell (1980) 20 : 761-767.
[13]Parent, C., Capelli, N., Berger, A., Crevecoeur, M., dan Dat, J.F. "An Overview of Plant Responses to Soil Waterlogging". Journal of Plant Stress (2008) 2 : 20-27.

[14]Hurng, W.P., Huu, S.L., Cherng, K.L., dan Ching, H.K.. "Role of absisic acid, ethylene, and polyamines in flooding promoted senescence tobacco leaves". Journal Plant Physiol (1993) 143: 102-105.
[15]Taiz, L dan E. Zeiger, *Plant Physiology: Fifth Edition*. Sunderland: Sinauer Associates, Inc. 2010.
[16]Hendriyani, I. K., dan N. Setiari. "Kandungan klorofil dan pertumbuhan kacang panjang *(Vigna sinensis)* pada tingkat penyediaan air yang berbeda". Jurnal Sains & Mat, Vol. 17(3) (2009) 145-150.
[17]Kurniawan, Bayu Adi., Sisca, Fajriani., dan Ariffin. "Pengaruh jumlah pemberian air terhadap respon pertumbuhan dan hasil tanaman tembakau".. Jurnal Produksi Tanaman (2014) 1 : 59-64.
[18]Laemmli, U.K. "Cleavage of structural protein during the assembly of the head of bacteriophage T4". Nature (1970) 227: 680-685.
[19]Bollag, D.M., dan Edelstein. S.J, *Protein Methods Department of Biochemistry*. Switzerland: University of Geneva Press 1991.
[20] Durrani, Rabia., Abubakar, M., Arshed, M. Javed., Ullah, S.S.I., dan Ali, Qurban. "Biological characterization and protein profiles of two model bacteria by SDS-Page and FT-IR". Journal of Agricultural and Biological Science Vol. 3 (5&6): (2008) 6-11.
[21]Stoychev, V., Lyudmila, S.S., Irina, V., Anelia, K.., Rosa, N., Urs, Feller., dan K. Demirevska. "Protein changes and proteolytic degradation in red and white clover plants subjected to waterlogging". Acta Physiol Plant 2013.
[22]Komatsu, S., dan Hossain, Z. "Organ-spesific proteome analysis for identification of abiotic stress response mechanism in crop". Frontiers in Plant Science Vol. 4 (2013).
[23]Meisrimler, C.N., Friedrich, B., dan Sabine, L. "Alterations in Soluble Class III Peroxidases of Maize Shoots by Flooding Stress". Proteomes (2014) 2: 303-322.
[24]Lüthje, S., Möller, B., Perrineau, F.C., dan Wöltje, K.. "Plasma membrane electron pathways and oxidative stress". Antioxid. Redox Signal (2013) 18: 2163–2183.
[25]Suzuki, N., Koussevitzky, S., Mittler, R., dan Miller, G. "ROS and redox signalling in the response of plants to abiotic stress". Plant Cell Environ (2012) 35: 259–270.
[26]Chang, R., Jang, C. J., Branco-Price, C., Nghiem, P., dan Bailey-Serres, J."Transient MPK6 activation in response to oxygen deprivation and reoxygenation is mediated by mitochondria and aids seedling survival in *Arabidopsis"*. Plant Mol. Biol (2012) 78: 109–122.

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