# INFLUENCE OF SALT STRESS ON *EX VITRO* GROWTH AND ANTIOXIDATIVE RESPONSE OF TWO *PAULOWNIA* CLONES

**K. Miladinova*, K. Ivanova**, T. Georgieva*, M. Geneva***, Y. Markovska****

*** Biotree, 8 Iliensko shoes str., 1220 Sofia, Bulgaria;**

** **Faculty of Biology, University of Sofia, 8 Dragan Tsankov Blvd., Sofia 1164, Bulgaria**

***** Istitute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. Georgi Bonchev str., bl. 21, Sofia 1113, Bulgaria**

**E-mail: pmmaster2001@yahoo.com**

*The morphological response of Paulownia tomentosa x fortunei* clone TF 01 *and Paulownia elongata x fortunei* clone EF 02, *grown in hydroponic at three levels of salinity, 50 mmol/l , 100 mmol/l, 200 mmol/l NaCl was compared. The content of malondialdehyde (MDA) was enhanced with increasing of salt stress. Activity of phenylalanine ammonia - lyase (PAL), which participate in the control of phenolic metabolism, was increased more in the leaves of Paulownia elongata x fortunei. Under salt stress phenolic and flavonoid contents were increased in the leaves of both clones selected. The results were discussed from the view of changes in synthesis of secondary metabolites and participation of separate classes of them in antioxidative response, characterizing different salt tolerance of Paulownia tomentosa x fortunei and Paulownia elongata x fortunei.*

***Key words**: salinity; growth; phenylalanine ammonia - lyase; phenols, flavonoids*

***Section: Biology***

High salinity is the most widespread abiotic stress and constitutes the most stringent factor in limiting plant distribution and productivity (Flowers et al., 1995:1). Salinity affects plant growth, metabolism and photosynthetic efficiency of crop plants (Maeda and Nakazawa, 2008:2; Misra et al., 1997:3). Much effort is being directed towards the identification of physiological and biochemical processes that are affected by NaCl in order to increase salt tolerance (Apse et al., 2002:4). Molecular approaches to target highly tolerant plants are also difficult as these plants frequently survive the stress periods, being in a state of suspended metabolism. The study of plant stress tolerance is suggested for understanding and transfer of tolerance traits to sensitive crop plants in future (Demmig-Adams et al., 2002:5).

*Paulownia* is native from China. *Paulownia tomentosa* has been introduced into USA and Europe as an ornamental plant and is still widely used for this purpose. Trees introduced in Bulgaria reach 12 m average height and 13.4 cm average diameter during 7 years (Kalmukov, 1995:6). Over the last two decades *Paulownia* species has been extensively studied due to its ability to uptake nitrates and land contaminants, namely heavy metals (Wang et al., 2010:7). This high-yielding tree can be used for the production of energy, paper pulp and wooden building materials. The genetically tissue–cultured *Paulownia* seedlings produced by The World Paulownia Institute (WPI) allow production of biofuels after introducing of cultivars without detrimental impacts on food supply or the environment. Research on *in vitro* propagation of *P. elongata* and *P. fortunei* has been reported (Bergmann et al., 1997:8). Application of this technology for micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production and it is effective way to maintain the genetic gain (Park and Bonga, 1992: 9).

Plants used in the current paper are propagated and rooted according technology registered of Biotree Ltd., Bulgaria. This laboratory is largest producer and supplier of genetically superior *Paulownia* tissue-cultures – *in vitro* seedlings. The farmers preferred *Paulownia tomentosa* x *fortunei* clone TF 01 due to fast development a uniform regular growth. *Paulownia elongata x fortunei* clone EF 02 is less branchy for the purpose of wood material formation. There is no information about its tolerance to salt stress and possibilities to improvement of saline soil

utilization with these clones. The results derived from tissue cultures can be used to predict the responses of plants to environmental contaminants and to improve the design and thus reduce the cost of subsequent conventional whole plant experiments (Doran, 2009:10).
In this research, the effect of NaCl on growth, activity of phenylalanine ammonia lyase, phenolic and flavonoid contents in leaves of *Paulownia tomentosa x fortunei* clone TF 01 and *Paulownia elongata x fortunei* clone EF 02, grown in hydroponic after transplant the explants were compared so as to provide fundamental base for vegetation restoration in contaminated soils.

## Materials and methods

**Plant material.** Seeds and *in vivo* explants from the species *P. tomentosa*, *P. elongata* and their hybrids with *P. fortunei* are used for developing of *in vitro* multiplication protocol. For induction of shoots, explants are cultured on Murashige and Skoog (MS) nutrient medium included 2.5% (w/v) sucrose, 0.8% (w/v) agar and vitamins. For a multiplication of shoots MS medium was used supplemented with 4.439 µM 6-benzylaminopurine (BAP) and 0.537 µM indolilacetic acid (IAA). The medium included 3.0% sucrose (w/v), 0.8% agar and vitamins. After multiplication the shoots were transferred to rooting medium based on half strength basal salts MS medium, 2 % sucrose, 6 % agar and vitamins supplemented with 4.92 µM indole-3-butyric acid (IBA) and 1.075 µM IAA. The pH of all media was adjusted to 5.7 using 0.1 N HCl and 0.1 N NaOH before autoclaving. All cultures were incubated under controlled conditions – 16 h photoperiod, light intensity of 35 µmol $m^{-2}$ $s^{-1}$ and 24/18±1ºC day/night temperature. After three weeks of rooting, the shoots were rinsed with 1.5 ml $L^{-1}$ Proplant solution.
**Hydroponic experiment.** The experiments were set as four treatments including control, each treatment with 3 replications. The uniform explants were selected and transplanted to polyethylene vessels containing 1.2 l of 1/4 Hellriegel solution (1898:11) with an addition of A-Z microelements after Hoagland (pH 5.9) in growth chamber with a 16-h photoperiod (PAR 100 µmol $m^{-2}$ $s^{-1}$ on the upper leaf surface, 25/23±1 ºC day/night temperature, relative humidity 60/70%). Each vessel contained two explants which represented one replication. After 21 days of cultivation the plants were transferred to 1/2 Hellriegel solution with the addition of A-Z microelements (pH 5.9). The salt treatment was applied on the 48$^{th}$ day after transplanting of explants when the plants had adapted to the conditions of 1/2 Hellriegel nutrient solution and 0 (control), 50, 100, and 200 mM/l NaCl was added. The solutions were aerated every day and were changed every 3 d to prevent depletion of nutrients and NaCl. Plants were harvested after 10 d of treatment. Toxicity symptoms (e.g. discoloration, pigmentation, yellowing and stunting) were assessed by eye through-out the experiment. At the end of the experiment the plant samples were collected, washed with tap water and rinsed with distilled water before being separated into leaf, petiole, stem and root and fresh mass of each plant sample were measured gravimetrically. Dry mass of shoots and roots were determined after oven-drying (60$^{o}$C) for 2 days until constant weight was obtained. Leaf area was calculated using software program.
**Enzyme and metabolite assays**. For measurements of phenylalanine ammonia-lyase (PAL), 0.2 g of FW of the third basal leaf of plants at each salt treatment were ground with 0.05 g PVP-40 into fine powder with liquid nitrogen in 4 ml of ice-cold buffer containing 100 mM potassium phosphate (pH 7.2), 2 mM EDTA, 4 mM DTT. The suspensions were centrifuged (16 000g, 20 min, 4$^{o}$C) (Yuan et al., 2002:12). Enzyme measurement of PAL (EC 4.3.1.24) activity was made: 0.1 ml supernatant was mixed with 0.25 ml 20 mM phenylalanine (dissolved in 100 mM borate buffer, pH 8.8), then 2ml of the same borate buffer and 1 ml of distilled water were added. Control samples contained the supernatant and buffer instead of phenylalanine. After 30, 60 and 90 min of incubation at 30ºC, absorption was measured at λ=290nm. Activity unit was calculated as ΔA = 0.01, equivalent to the production of 3.09 nmol

cinnamic acid (Yuan et al., 2002:12). Protein content was measured with Folin reagent (Lowry et al., 1951:13). For determination of malondialdehyde (MDA), 0.3 g FW of the third basal leaf of plants at each salt treatment were homogenized in a mortar at 4°C with 3 ml 0.1 % trichloroacetic acid and centrifuged for 20 min at 15 000 rpm. 0.5 ml of the supernatant were mixed with 0.5 ml phosphate buffer pH 7.4 and after the addition of 1 ml 0.5 % thiobarbituric acid dissolved in 20 % trichloroacetic acid, the samples were boiled for 30 min (Dhindsa et al., 1981:14). After rapid cooling of the samples in an ice-bath, absorption was measured at $\lambda$=532 and 600nm using the extinction 3oefficient 155 $mM^{-1}$ $cm^{-1}$ (Heath and Packer 1968:15). For determination of the phenols and flavonoids, dry samples (100 mg) from third basal leaf of plants at each salt treatment were ground and exhaustively extracted with 96% (v/v) methanol. Total phenolics were determined by the Folin & Ciocalteu's colorimetric method (Pfeffer et al.,1998:16), modified by us as follows: an aliquot of the extract was placed in test-tube and distilled water, 1:1 Folin & Ciocalteu's reagent and 20% $Na_2CO_3$ were added. The absorbtion was measured at $\lambda$=730 nm and the total phenolics were calculated by means of a calibration curve of chlorogenic acid (in the range of 30µg/ml to 100 µg/ml) and expressed as mg of chlorogenic acid equivalent per 1 g DW of the sample. Total flavonoids content of the whole shoot samples of the plant was measured using a colorimetric assay (Zhishen, 1999:17), modified as follows: Aliquots of the methanol extract were placed in test-tube and distilled water, 5% $NaNO_2$ and 10% $AlCl_3$ were added. After the addition of 1N NaOH and distilled water, the absorption at $\lambda$=510 nm was measured and the concentration was calculated by means of a calibration curve of (+) catechin (in the range of 2 µg/ml to 80 µg/ml). The total flavonoids of the samples were expressed in mg of (+) catechin equivalent per 1 g DW of the sample. All measurements were performed in triplicate with three repetitions.

**Results and disccusion**

**Effect of salt stress on plants growth.** Seedlings growth is normally limited by increasing concentration of NaCl (Sreenivasulu et al., 2000:18). In our study, with increasing salinity levels, the root and stem length, leaf number and total leaf area in both plants were reduced (Table 1). The root and stem length of *Paulownia tomentosa x fortunei* clone TF 01 was reduced more than that of *Paulownia elongata x fortunei* clone EF 02. The values for stem length measured at 200 mM/l NaCl for *Paulownia elongata x fortunei* clone TF 01 were close to the control. The leaf number of *Paulownia tomentosa x fortunei* clone EF 02 rose, but total leaf area declined sharply with increasing salinity levels. Maximum reduction of total leaf area was observed at 200 mM/l NaCl (80%).The leaf number of *Paulownia elongata x fortunei* clone was the same at 100 and 200 mM/l NaCl. With increasing salinity levels total leaf area was reduced by 40%; 28% and 36%, respectively, compared to control. Total leaf area showed the capability of a plant in forming of photosynthetic surface. The total FW/DW per plant was increased for *Paulownia tomentosa x fortunei* by 21%; 13% and 47%, respectively, but was decreased for *Paulownia elongata x fortunei* by 2%; 12% and 5%, respectively, compared to control (data not showed). The present results obtained for *Paulownia elongata x fortunei* were in line with those of Parveen and Farrukh (2009:19), who reported decline in fresh and dry weights of shoots under high salinity stress.

**Effect of salt stress on plants malondialdehyde**. The increased accumulation of lipid peroxides is indicative of enhanced production of toxic oxygen (Markovska et al., 2009:20). In this study, MDA concentration was enhanced with increasing salinity levels, the maximum values was observed at 200 mM/l NaCl (Table 2). The results indicated that salt stress produced more reactive oxygen species, resulting in more increased lipid peroxidative products and oxidative stress in *Paulownia tomentosa x fortunei* clone than in *Paulownia elongata x fortunei* clone.

**Effect of salt stress on plants secondary metabolism.** PAL is involved in the control of phenolic metabolism and provides precursors for lignin biosynthesis (Hahlbrock and Scheel, 1989:21). ). It is known that the level of PAL are affected by age, light, phytochrome, wonding, infection (Camm and Towers, 1973:22).Our results showed that PAL activity in the leaves of *Paulownia elongata x fortunei* was three time higher compared to that in the leaves of *Paulownia tomentosa x fortunei* (Fig. 1). PAL activity was enhanced in both plants with increasing salinity levels. Flavonoids and phenolic substances isolated from wide range of vascular plants, act in plants as antioxidants and antimicrobials (Pietta, 2000:23). Shukla et al. (2009:24) reported about a significant and linear relationship between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to antioxidant activity. Our results showed that PAL activity, phenolic and flavonoid content rose with increasing salinity levels. The maximum values were observed at 100 and 200 mM/l NaCl. There is some evidence of the induction of phenolic metabolism in plants as a response to multiple stress (including salt stress) (Michalak, 2006:25). Phenolics including various flavonoids play pivotal roles in absorbing free radicals, quenching singlet oxygen, and decomposing peroxides. *Paulownia elongata x fortunei* clone TF 01 is more tolerant to salt stress than *Paulownia tomentosa x fortunei* clone EF 02. It is characterized with smaller reduction of root and stem length, total leaf area and higher PAL activity, total phenolic and flavonoid content in the leaves.

**Table 1.** Mean values ± SD (n = 5-6) of root and stem length, leaf number and total leaf area of *Paulownia tomentosa x fortunei* clone TF 01 and *Paulownia elongata x fortunei* clone EF 02, grown in hydroponic in response to salt stress

| Treatments | Root length [cm] | Stem length [cm] | Leaf number | Leaf area [$cm^{2}$] |
|---|---|---|---|---|
| *Paulownia tomentosax fortunei* | | | | |
| Control | 28.25±2.12b | 8.57±0.91b | 12±1.7b | 429±16b |
| 50 mM/l NaCl | 19.06±2.87a | 6.13±0.71a | 8±0.6a | 126± 5a |
| 100 mM/l NaCl | 21.71±3.29a | 6.53±0.64a | 9±1.5a | 109±22a |
| 200 mM/l NaCl | 18.31±3.56a | 5.63±0.66a | 11±1.5b | 87± 4a |
| *Paulownia elongate x fortunei* | | | | |
| Control | 37.51±2.12b | 10.51±0.51b | 10±0.8a | 502±39b |
| 50 mM/l NaCl | 26.81±1.85a | 8.41±0.33a | 9±1.1a | 300±40a |
| 100 mM/l NaCl | 26.71±1.61a | 9.11±1.91a | 8±1.2a | 360±23a |
| 200 mM/l NaCl | 27.71±1.67a | 10.11±1.51b | 8±0.7a | 321±67a |

Values with the same letter are not significantly different when means are separated by Fisher`sLSD test ($P<0.05$).

**Table 2.** Mean values ± SD (n = 5-6) of MDA content (in nM/gFW), determined in leaves of *Paulownia tomentosa x fortunei* clone TF 01 and *Paulownia elongata x fortunei* clone EF 02, grown in hydroponic in response to salt stress

| Treatments | *Paulownia tomentosa x fortunei* | *Paulownia elongata x fortunei* |
|---|---|---|
| Control | 0.017±0.000b | 0.017±0.000b |
| 50 mM/l NaCl | 0.298±0.005a | 0.146±0.021a |
| 100 mM/l NaCl | 0.281±0.034a | 0.128±0.024a |
| 200 mM/l NaCl | 0.396±0.031c | 0.191±0.013c |

Values with the same letter are not significantly different when means are separated by Fisher`s LSD test (P<0.05).

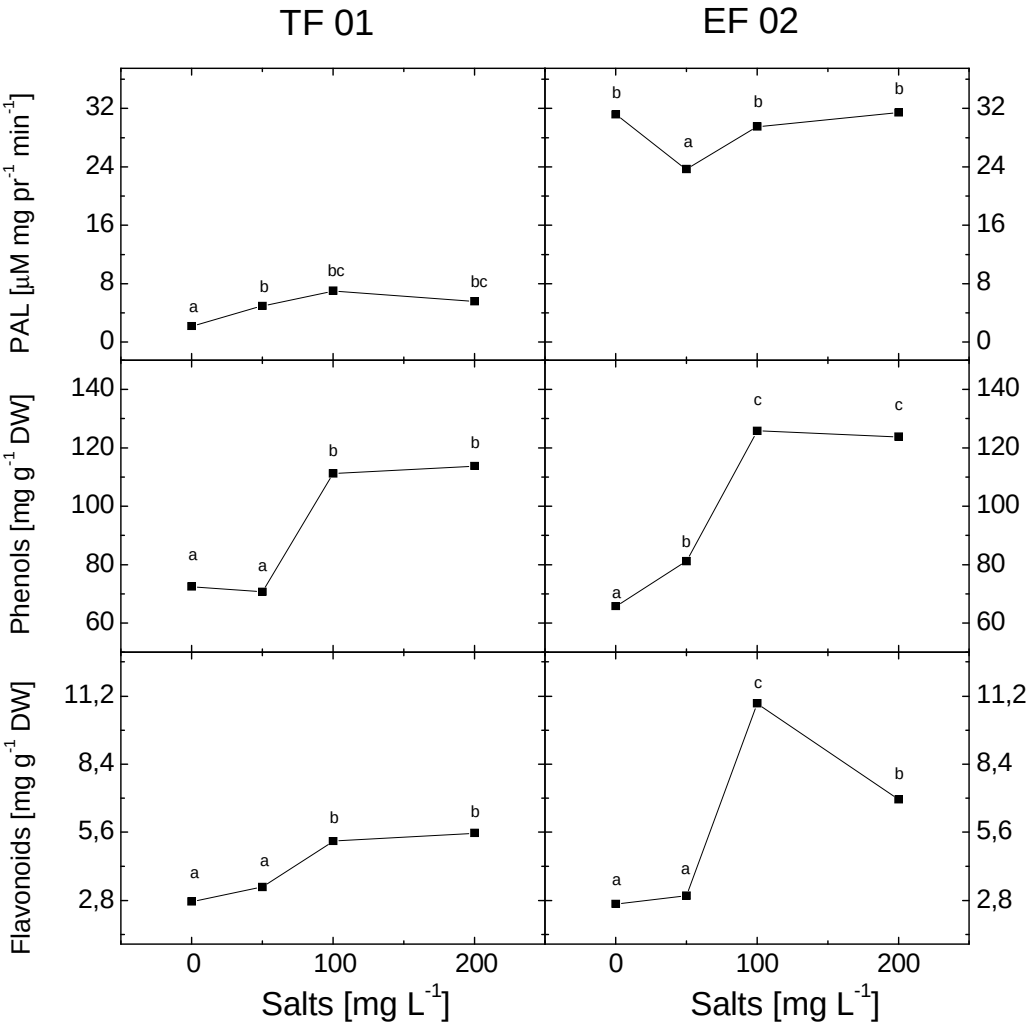


**Figure 1.** Changes in the PAL activity, phenol and flavonoid content, determined in leaves of *Paulownia tomentosa x fortunei* and *Paulownia elongata x fortunei* clones, grown in hydroponic in response to the addition of NaCl

The tolerance of *Paulownia elongata x fortunei* clone TF 01 for high-salinity environments makes it a possible candidate for studying the molecular mechanisms by which plants respond to salinity stress. Changes in the levels of some nonenzymatic antioxidants, such as phenolics and flavonoids may estimate for their use as markers of salt tolerance in genetically diverse *Paulownia* clones.

**References**


1. Flowers, T.J., Yeo, A.R. 1995. Breeding for salinity tolerance in crop plants. Where next? J. Plant Physiol., 22, 875-884.

2. Maeda, Y., Nakazawa, R. 2008. Effects of the timing of calcium application on the elevation of salt stress in the maize, tall fescue, and reed canarygrass seedlings. Biol. Plant., 52, 153-156.

3. Misra, A.N., Sahu, S.M., Misra, M., Singh, P., Meera, I., Das, N., Kar, M., Sahu, P. 1997. Sodium chloride induced changes in leaf growth, and pigment and protein contents in two rice cultivars. Biol. Plant., 39, 257-262.

4. Apse, M.P., Blumvald, E. 2002. Engineering salt tolerance in plants. Curr. Opin. Biotechnol., 13, 146-150.

5. Demmig-Adams, B., Adams, W.W. 2002. Antioxidants in photosynthesis and human nutrition. Science, 298, 2149-2153.

6. Kalmukov, K. 1995. Influence of primary density upon the process of growth and development of some fast growing tree species used for biomass. In: Brezin, V.N. (ed.) 70 Years Forestry education in Bulgaria. Jubilee Sciences Session, 7-9.VI. 1995. Sofia, v. I, 129-137.

7. Wang, J., Li, W., Zhang, C., Ke, S. 2010. Physiological responses and detoxific mechanisms to Pb, Zn, Cu and Cd in young seedlings of *Paulownia fortunei*. J. Environ. Sci. (China), 22, 1916-1922.

8. Bergmann, B.A., Moon, H.-K. 1997. In vitro adventitious shoot production in *Paulownia*. Plant cell reports, 16, 315-319.

9. Park, Y.S., Bonga, J.M. 1992. Conifer micropropagation: its function in tree improvement programs. In: Ahuja, M.R. (ed.) Micropropagation of woody plants. Kluwer Academic, Dordrecht, pp 457-470.

10. Doran, P.M. 2009. Application of plant tissue cultures in phytoremediation research: Incentives and limitation. Biotechnol. Bioeng., 103, 60-76.

11. Hellriegel, H.1898. Die methode der sandkulture. Arb. Landwirtsch. Ges., 34, 15-18.

12. Yuan YJ, Li C, Hu ZD, Wu JC, Zeng AP (2002) Fungal elicitor-induced cell apoptosis in suspension cultures of *Taxus chinensis* var. *mairei* for taxol production. Process Biochem., 38, 193-198.

13. Lowry, O.H., N.J. Rosenbough, A.L. Farr, R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265-275.

14. Dhindsa, R., Plunb-Dhindsa, T., Thorpe, T. 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot., 32, 93-101.

15. Heath, R.L., Packer, L. 1968. Phoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys., 125, 189-190.

16. Pfeffer, H., Dannel, F., Römheld, V. 1998. Are there connection between phenol metabolism, ascorbate metabolism and membrane integrity in leaves of boron-deficient sunflower plants? Physiol. Plantarum, 104, 479-485.

17. Zhishen, J., Mengcheng, T., Jianming, W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem., 64, 555-559.

18. Sreenivasulu, N., Grimm, B., Wobus, U. 2000. Differential response of antioxidant compounds to salinity stress in salt tolerant and salt sensitive seedlings of foxtail millet (*Setaria italica*). Physiol. Plant., 109, 435-443.

19. Parveen, A., Farrukh, H. 2009. Growth performance of *Vicia sativa* under saline conditions. Pakistan J. Bot., 41, 3075-3080.

20. Markovska, Y.K., Gorinova, N.I., Nedkovska, M.P., Miteva, K. 2009. Cadmium-induced oxidative damage and antioxidant response in *Brassica juncea* plants. Biol. Plant., 53, 151-154.
21. Hahlbrock, K., Scheel, D. 1989. Physiology and molecular biology of phenylpropanoid metabolism. Ann. Rev. Plant Phys. Plant Mol. Biol., 40, 347-369.
22. Camm, E.L., Towers, G.H.N. 1973. Phenylalanine ammonia lyase. Phytochemistry, 12,961-973.
23. Pietta P.G. 2000. Flavonoids as antioxidants, J. Nat. Products, 63, 1035-1042.
24. Shukla S., Mehta A., Bajpai V.K., Shukla S. 2009. *In vitro* antioxidant activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* Bert, Food Chem. Toxicol., 47, 2338–2343.
25. Michalak, A. 2006. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Pol. J. Environm. Stud., 15, 523-530.

**Yuliana Kostadinova Markovska**

**Associate Prefessor**

**Faculty of Biology, University of Sofia, 8 Dragan Tsankov Blvd., Sofia 1164, Bulgaria**
**Tel.: 02 8167 265**

**GSM: 0884 272 883**

**E-mail: pmmaster2001@yahoo.com**