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In vitro effect of plant growth regulators (PGRs) for callus induction and plant regeneration from suspension of Hamata (*Stylosanthes hamata* cv. Verano)

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Abstract The plant regeneration from seed of Hamata (*Stylosanthes hamata* cv. Verano) was found. Seeds were induced on MS medium (Murashige and Skoog, 1962) with different concentration of cytokinin were used 0.5, 1 and 3 mg/L 6-benzyladenine (BAP) *meta*-Topolin (*m*T) or Thidizuron (TDZ), respectively. The result showed that the highest percentage of seed induction was 65% and average shoots 8.66 shoots per seed on MS medium with 3 mg/L *m*T for 12 weeks cultivation. For node of hamata were study on callus and shoot induction on MS medium with 0.5 mg/L IBA combined with 0.5, 1 and 3 mg/L TDZ. The highest percentage of callus and shoot induction was 100% gave average areas of callus at 11192.84 mm^3 and the number of shoots per callus was 13.50 shoots on MS medium with 0.5 mg/L IBA combined 3 mg/L TDZ for 8 weeks cultivation Cell suspension culture of callus that developed from node of hamata. The friable callus was transferred to liquid MS medium with 0.5 mg/L IBA combined with 3 mg/L TDZ for 4 weeks cultivation After that transferred to MS medium with 3 mg/L *m*T for shoots regeneration. The percentage of shoots generation from callus was shown on 90% and the number of shoots per callus was 14.00 shoots for 12 weeks cultivation. Transferred shoot to root medium containing of MS medium with Indole-3-butyric acid (IBA) Naphthaleneacetic acid (NAA) or Indole-3-acetic acid (IAA) at 0.1, 0.3 and 0.5 mg/L. The result showed that the highest percentage of root induction was 100% and gave averaged roots at $11.00^{\pm}1.87$ roots per shoot on MS medium with 0.3 mg/L NAA for 12 weeks cultivation.

Keywords: Node, Plant Growth Regulators suspension, *Stylosanthes hamata* cv. Verano

Introduction

Legumes a large family of plants from the grass. It is important for human and animal food. The plants are high nutritious and protein. In addition

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to food, Fabaceae plant has significant to economic because Fabaceae plant are special from other crops. It can use nitrogen in the air to growth. *Rhizobium* causes root-knot obtain food and energy from plant. while Fabaceae plant obtain compounds product from rhizobium use to growth. So, legume plants are important role in maintaining soil fertility decrease soil erosion. In addition, legume used in "ley-farming" to increase the soil abundance. Some legume species play a role in forest and used in biofuel (Jones *et al*., 1997).

Hamata (*Stylosanthes hamata* cv. Verano), a native bean from Venezuela in South America, was been studied by Khonkaen University since 1970 by Dr. Shelton and Dr. Humphreys, Queenlands University, Australia. Hamata can be growth on normal weather in Northeast. High protein content and resistant to grazing animals. There are no reports that make beef cattle and dairy cattle flatulence like some beans. And high seed yield (Bureau of Animal Nutrition Development, 1982). In tests comparing varieties at KhonKaen University. Hamata produced the higher yield than forage variety. According to (Topark-Ngarm *et al*. (1979). Seed production has been tested to find the maximum yield of seed. For example, the optimal time which would release the beef cattle and dairy cattle before harvesting the seeds or harvest the seeds before cutting the bean (Wilaipon and Humphreys, 1976; Wilaipon *et al*., 1979; Wilaipon and Humphreys, 1981).

The report is available on tissue culture of *Stylosanthes* spp. using cotyledon and hypocotyl induction callus on MS medium with BAP and NAA in different concentrations were used for callus induction and shoots regeneration. The percentage of cotyledon left and hypocotyl from callus of *Stylosanthes* spp. were 90.33% and 81.33%, respectively. on MS medium with 3.0 mg/L BAP and 1 mg/LNAA (Kumar and Chandra (2010) According to, (Boonrung *et al*., 2012) studied of cell suspension of *Stylobates guianesis* CIAT 184 by using the callus derived from cotyledon callus induction on MS medium with 1 mg/L 2,4-D. The results showed fresh weight and dry weight of cell suspension with the best growth for 15 days of 0.6304 g/25mL and 0.0360 g/25mL, respectively.

The aims of this study were to investigated *in vitro* callus induction and cell suspension regeneration from node with optimum and concentration of plant growth regulators for *Stylosanthes hamata* cv. Verano.

Materials and Methods

Plant material and plant regeneration

The used seeds of hamata (*Stylosanthes hamata* cv. Verano) were supported by Feed and Forage Analysis Section, Animal Nautrition Division,

Department of Livestock Development, Pathumthani Province, Thailand. Seeds of hamata were surface sterilized by 70% ethanol for 2 min and 15% sodium hypochlorite solution with few drops of tween 20 for 15 min and washed 3 times in sterilized water. The seeds were placed on 3 different Murashige and Skoog (1962) media with various concentrations at 0.5, 1, and 3 mg/L, 6-benzlyaminopurine (BAP), Thiazine (TDZ) and *meta*-Topolin (*m*T) Medium containing 30 g/L sucrose and 2.6 g/L phytagel (pH 5.8). The culture was incubated at 25 ±1 ˚C under fluorescent light (27 µmol $m^{-2}s^{-1}$) for 16hrs and dark condition for 8 hrs.

Callus induction

Callus was derived from hamata node and obtained regeneration on MS medium containing combinations of 0.5 mg/L Indole-3-butyric acid (IBA) and TDZ (0.5, 1 and 5 mg/L). Callus was subculture on a new medium every 4 weeks cultivation. Callus composition and structure was observed under Field Emission Scanning Electron Microscope and Energy Dispersive X-ray Spectrometer (FESEM-EDS (7610F)).

Suspension culture

The friable callus was transferred into liquid MS medium of 0.5 mg/L IBA combined with 3 mg/L TDZ for 12 weeks cultivation. The culture was maintained at 120 rpm 25±1 ℃. After suspension culture made shoots were on MS medium with 3 mg/L *m*T.

Roots induction

The shoots from 12 weeks cultivation regenerating callus were taken to the MS medium with Indole-3-butyric acid (IBA) Naphthaleneacetic acid (NAA) or Indole-3-acetic acid (IAA) at 0.1, 0.3 and 0.5 mg/L.

Data analyses

All treatments were repeated three replicates. ANOVA was involved using SPSS v25.The data was analyzed by one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) for mean comparison at *p*-values 0.05

Results

Plant regeneration

The seeds were regenerated to multiple shoots culture with 0.5, 1, and 3 mg/L BAP, TDZ and *m*T, respectively. The percentage of germination was 65%. The results for multiple shoots from cultured seeds with 3 mg/L *m*T were 8.66 shoots per seed. After 8 weeks cultured, shoots length was 4.28±0.10 cm. (Figure 1A) The lowest of shoots regenerated was 1.00 shoots per seed and shoots length was 0.47 cm was recorded in 0.5 mg/L BA (Table 1).

Callus induction from node

The formatted callus could be observed from the node after 4 weeks cultivation seeding responded with 0.5 mg/L IBA combined with 1, 3 and 5 mg/ml TDZ (Table 2). The formatted callus was compact, green (Figure 1B) and friable callus were yellow. (Figure 2A) However, the callus was friable on with 0.5 mg/L IBA combined with 3 mg/L TDZ. The maximum percentage of callus was 77% observed in 0.5 mg/L IBA combined with 3 mg/L were 13.5±0.14 shoots per callus and shoots length was 1.30±0.14 cm for 8 weeks cultivation. However, the callus percentage was 55% on 0.5 mg/L IBA combined with 5 mg/L TDZ. The highest compact callus per callus was 19712.08 mm^3 (Table1). The callus structure from hamata node by SEM resolution 500 mm at 15 kv and magnification x15 (Figure 1C).

Shoot regeneration from suspension

The friable callus was transferred to liquid MS medium with 0.5 mg/L IBA combined with 3 mg/L TDZ. The callus was changed from yellow to green after 4 weeks cultivation (Figure 2B) The embryogenic callus initiated to multiple shoots for 8 weeks cultivation. (Figure 2C). After that transferred to MS medium with 3 mg/L *m*T for shoots regeneration. The percentage of shoots generation from callus was 90%. The shoots per callus was 14.00 shoots and shoots length were 1.24±0.08 cm for 8 weeks cultivation. (Figure 2D).

Root induction from the regenerated shoots

The roots were cultured with 0.1, 0.3, and 0.5 mg/L IBA, IAA and NAA, respectively (Table 3). The roots were initiated after 2 weeks cultivation. The roots per shoot, roots length was measured after 12 weeks cultivation. The maximum of roots formation in 0.3 mg/L NAA were 11.00±1.87 roots per root and roots length was 2.84±1.63 cm after 8 weeks cultivation (Figure 2E). The

lowest of regenerated roots were 2.50±0.64 roots and roots length was 0.13±0.26 cm with 0.1 mg/L IBA.

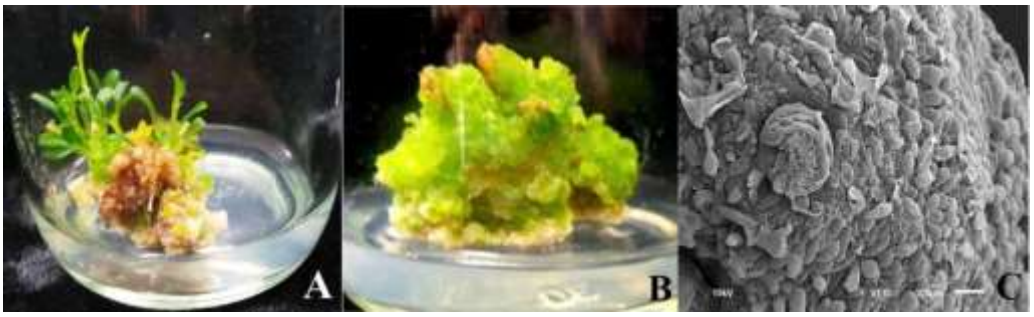

Figure 1. Induction of shoots in Hamata (*Stylosanthes hamata* cv. Verano) culture (A) Multiple shoot induced from seed with 3 mg/L *m*T for 8 weeks (B) Compact callus induction from node with 0.5 mg/L IBA and combined with 5 mg/mL TDZ for 8 weeks (C) The callus structure from hamata node by SEM resolution 500 mm at 15 kv and magnification x15

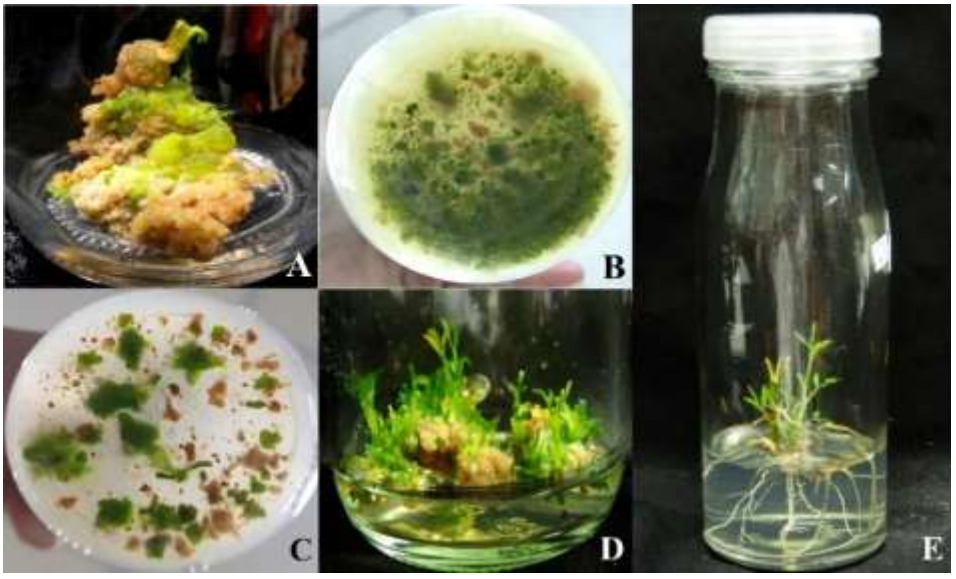

Figure 2. Callus and suspension cultures of *S. hamata* cv. Verano (A) Friable callus induction from node with 0.5 mg/L IBA and combined with 3 mg/mL TDZ for 8 weeks (B-C) Cell suspension induction with 0.5 mg/L IBA and combined with 3 mg/mL for 4 and 8 weeks, respectively (D) Multiple shoots regenerated from suspension culture with 3 mg/L *mT* for 12 weeks (E) The roots induction with 0.3 mg/L NAA for 12 weeks

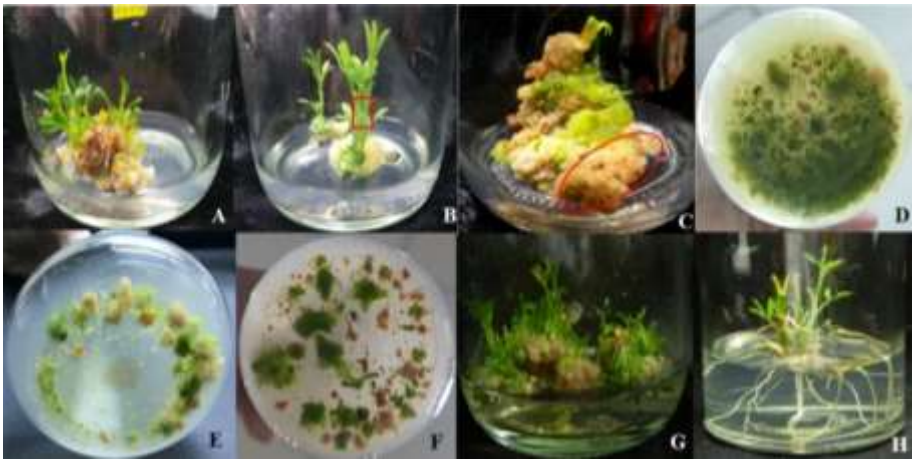

Figure 3. Step for shoots and roots induction of *S. hamata* cv. Verano (A) Multiple shoot induced from seed on MS medium with 3 mg/L *m*T for 8 weeks (B) The node with 2-3 cm (C) Callus induction from node with 0.5 mg/L IBA combined with 3 mg/mL TDZ for 8 weeks. (D-F) Cell suspension induction with 0.5 mg/L IBA and combined with 3 mg/mL for 4, 8 and 12 weeks, respectively. (G) Multiple shoots regenerated from suspension culture with 3 mg/L *mT* for 12 weeks (H) The roots induction with 0.3 mg/L NAA for 12 weeks

Table 1. Effects of different concentrations of BAP, TDZ and *m*T in MS medium on multiple shoots induction from seed of *Stylosanthes hamata* cv. Verano

Plant growth regulators (mg/L)			**The number of initial seeds**	**Shoot formation (%)[1/]**	**Shoot/seed explants[2/]**	**Shoots length (cm)**
BAP	TDZ	*m*T				
	control		80	0	0	0
0.5	0	0	80	60.00	1.00 ±0.0^{f}	0.0±47.23^{b}
1	0	0	80	57.50	4.±500.23^{b}	0.0±71.40^{b}
3	0	0	80	45.50	1.±530.01^{e}	0.0±47.06^{b}
0	0.5	0	80	56.25	1.33 ±0.01^{e}	0.0±31.01^{b}
0	1	0	80	60.00	1.41 ±0.26^{e}	0.0±48.15^{b}
0	3	0	80	51.25	4.±330.01^{b}	1.0±45.06^{b}
0	0	0.5	80	55.00	2.00 ±0.00^{d}	0.0±26.35^{b}
0	0	1	80	63.75	4.00 ±0.00^{c}	5.1±03.35^{a}
0	0	3	80	65.00	8.±660.04^{a}	4.0±28.10^{a}

[a-f] Means in same column with different letters are significantly different at P < 0.5 depending on DMRT using one-way ANOVA

[1/] Seeds germination means germinated seeds out of total seeds number

[2/] Shoots growth initiation means developed shoots out of total seeds number

Table 2. Effects of different concentrations of IBA in combination with optimized concentrations of TDZ in MS medium on callus induction from node of *Stylosanthes hamata* cv. Verano

Plant growth regulators (mg/L)		The number of initial nodes	Callus formation (%)[1/]	Average callus number per node mm^3	Shoot/node explants[2/]	Average length of shoot (cm)
IBA	TDZ					
0.5	1	40	77	8111.99	2.±250.19^{c}	0.610±.19^{c}
0.5	3	40	100	11192.84	13.5 ±0.14^{a}	1.300±.14^{b}
0.5	5	40	55	19712.08	6.50 ±0.06^{b}	0.0±76.06^{a}

[a-b] Means in same column with different letters are significantly different at P < 0.5 depending on DMRT using one-way ANOVA
[1/] Callus germination means germinated node out of total node number.
[2/] Shoot growth initiation means developed shoots out of total node number

Table 3. Effects of different concentrations of IBA, IAA and NAA in MS medium on roots induction from shoots of *Stylosanthes hamata* cv. Verano

Plant growth regulators (mg L^{-1})			The number of initial seeds	Root formation developed (%)[1/]	Roots/ explants[2/]	Roots length (cm)
IBA	IAA	NAA				
control	0	0	10	0	0	0^{g}
0.1	0	0	10	40	2.50±0.64^{de}	0.13±0.26d^{e}
0.3	0	0	10	50	4.25±0.25^{cd}	0.15±0.33^{c}
0.5	0	0	10	80	10.25±2.13^{a}	2.26±0.50^{b}
0	0.1	0	10	50	6.50±0.86^{bc}	1.35±0.24^{cd}
0	0.3	0	10	70	5.50±0.86^{cd}	2.16±0.59^{b}
0	0.5	0	10	30	2.75±1.18^{de}	1.30±0.34^{de}
0	0	0.1	10	100	9.00±0.70^{ab}	1.15±0.90^{ef}
0	0	0.3	10	100	11.00±1.87^{a}	2.84±1.63^{a}
0	0	0.5	10	100	2.75±0.85^{de}	1.07±0.29^{f}

[a-f] Means in same column with different letters are significantly different at P < 0.5 depending on DMRT using one-way ANOVA
[1/] Roots germination means germinated shoots out of total node number.
[2/] Roots growth initiation means developed shoots out of total node number

Discussion

Hamata seeds were induced with different concentration of cytokinin were 0.5, 1 and 3 mg/L BAP, *mT* and TDZ, respectively. The result showed the highest percentage of seed induction was 65% and shoots were 8.66 shoots per shoots with 3 mg/L *m*T after 12 weeks cultivation. The previous report of micropropagation from seeds (Boonruang *et al*., 2011) explained that 1 mg/L

BAP shoots were 5.06 shoots per seed with 1 mg/L BAP for 8 weeks cultivation. In the present study, the best growth regulator was 3 mg/L. *m*T made seeds multiple induction were 8.66 shoots per seed and shoots length was 4.28 cm after 8 weeks cultivation. *m*T was a high performance for seeds germination on *Stylosanthes. hamata* Amoo *et al*. (2010) reported that high concentration of *m*T had less toxic than higher concentrations of BA.

The callus was regenerated from node with 0.5 mg/L IBA combined with 3 mg/mL TDZ was the best regeneration. Similar to Movahedi *et al*. (2015) studied on plant growth regulators, cotyledon and epicotyl cultured with 0.5 mg/L IBA combined with 3 mg/L TDZ. The highest callus had fresh weight 3.15 g and TDZ combined with IBA was the best treatment for callus induction, the results are like Mustafa *et al.* (2017) callus induction of *Phaseolus vulgaris* L. with 2.5 mg/L 2,4-D combined with 0.5 mg/L BAP. The shoots per callus was 3.00 shoots and shoots length were 16 cm. In the other hand, Hussaini *et al*, (2015) suggested that the callus induction and plant regeneration in potato (Arnova) with 0.22 mg/L TDZ combined with 0.49 mg/L NAA The Shoot formation completely failed after second and third subculture The TDZ and NAA were better response for cell division when higher concentration of auxin and cytokinin applied together.

Cell suspension culture was friable callus and developed to compact callus. The result was agreed with Gruel *et al*. (2001) in resulted that TDZ stimulated a compact-green callus from node. Al-Juboory *et al.* (1998) and Murthy *et al*. (1998) suggested MS medium with 2.20 mg/L TDZ combined with 0.44 mg/L IAA was more effective for callus production from gardenia. The efficacy of TDZ was strongly supported callus induction from shrub plant. More recently, TDZ induced shoots regeneration better than BAP in *Arachis hypogaea* because TDZ had the morpho-regulatory potential of the chemical that led to tissue and organ culture application (Victor *et al*., 1999).

For roots induction use auxin (IBA, IAA and NAA) in MS medium. The result showed the highest percentage of roots induction was 100% and roots formation at 11.00$\pm$1.87 roots per shoot with 0.3 mg/L NAA for 12 weeks cultivation. Similar to Rey *et al*. (2000) studied on microshoots of *Arachis pintoi*. The best roots induction used with 0.01 mg/L NAA. Morever, Dolce *et al*. (2017) suggested that the root induction with 1 g/L of activated carbon (AC). In the field, *Arachis pintoi* was acclimatized and made 90% of roots induction. In the future of this study cell suspension of hamata can be new mutation by gamma irradiation for salt tolerant and drought tolerant.

It concluded that seeds of *Stylosanthes hamata* cv. Verano were induced 3 mg/L *mT*. The result showed the highest percentage of seed induction was 65% and average shoots 8.66 shoots per shoots at 12 weeks cultivation. The callus

induction with 0.5 mg/L IBA combined with 3 mg/L TDZ. The highest percentage of callus induction was 100% gave average areas of callus as 11192.84 mm^3. The percentage of shoots generation by cell suspension from callus was 90% and the shoots per callus was 14.00 shoots at 12 weeks cultivation. The roots induction with NAA 0.3 mg/L. The result showed the highest percentage of root induction was 100% and average of roots were 11.00$\pm$1.87 roots per shoot.

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Hamata (*Stylosanthes hamata* cv. Verano) were received from Feed and Forage Analysis Section. Animal Nutrition Division. Department of Livestock Development, Pathumthani Province, Thailand. This work supported by Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL).

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