IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 11, Issue 10 Ver. I (October 2018), PP 51-56
www.iosrjournals.org

In Vitro responses of Wild Blackberries (Rubus Spp.) tosterilization protocols, Different Parts of Plant and Sizes of Explant Used in Micro Propagationin Kenya

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Abstract: *Blackberry is a crop of great economic potential due to its health benefits to humans. Despite the economic importance, it has not been exploited in Kenya. Eighty-four species have been identified in the country but there is little information on propagation about the crop. With enough research blackberry can be adopted for commercialization. To ease Commercialization, a quick propagation procedure needs to be developed. In the present study, sterilization, different plant part explants and size of explant were evaluated inRubus fruiticous, Rubus apatelus and Rubus volkensis.Complete Randomized Design composed of three replications per treatment was used. Data were subjected to analysis of variance (ANOVA) using the general linear model procedure of the statistical analysis system (SAS) program (SAS institute I.nc, 2007). Significant means were separated using Tukey's honestly significant deference (Tukey's HSD) test (P≤ 0.05). For sterilization experiments, a wash with tap water, Caberndezim for 10min, 5% NaOCl for 2 min and 70% ethanol for 2 min gave the least contamination of 11.11% and the highest survival rate of explants per vessel of 5.111 explants per culture vessel at 5% level of significance. Nodal sections from apical sections had the highest survival rates of explants per vessel with Rubus fruiticosus giving 100% survival. Between the wild species Rubus volkensisa wild species responded better while Rubus apatelus had 0.000 explants per vessel. On the different explant sizes tested for the species apatelusthere were significant effects on survival rates in which size 2.1-3.0 cm had highest survival rate of 93.333%.*

Key words*: Micropropagation, in vitro, wild, blackberry*

Date of Submission: 12-10-2018 Date of acceptance: 27-10-2018

I. Background Information

Small berry fruits are normally consumed because of their rich supply of nutrients e.g. , and antioxidants, bright and attractive colors, and their special taste. Berry fruits include; blueberry, blackberry, currants,gooseberry, strawberry, raspberry and cranberry (Finn and Clark, 2011). The crop is a shrub with erect, semi-erect or creeping growth habit, and most cultivars have thorny stems. Blackberry belongs to the fruiting plants of theRosaceae family. The fruit contains high and significant amounts of phenolic flavonoid phytochemicals such as anthocyanins, allagic tannin, guercetin, catechnis, kaempferolgallic acid, cyanidins, pelargonidinsand salicylic acid.

Plant biotechnology is an achievement of science and technology in the recent world and it has a huge role in the development and progress of modern horticulture in production of food and nutritional security needs. The technique of growing sterile plant cells, tissue or organs separate from the mother plant on artificial medium is called plant tissue culture. Plant tissue culture i.e. micropropagation overs an option for a quick and rapid production process for genetically identical plant individuals using a small amount of resources, space and time (Odutayo*et al*., 2004;Fira*et al*., 2014).Plant tissue culture success has been reported for many members of the genus Rubus involving callus cultures, shoot tips growth,parthenocarpic fruit and roots development(Skirvin*et al*., 1981)

Aseptic conditions are usually practiced in plant tissue culture for the successful establishment and maintenance of plant cell, tissue and organ culture. Microbes can be present in the explants or can be reintroduced during handling due to unhygienic conditions in the laboratory, the operator or from laboratory instruments.The mostconstant problem in tissue culture is microbial contamination which compromises development of in vitro cultures(Webster *et al*., 2003). Microbes compete with plant tissue cultures for nutrients, resulting in increased culture mortality. Also tissue necrosis, variable growth, reduced shoot proliferation and reduced rootinghave been reported (Oyebanji*et al*., 2009). It is important to establish sterile explants for in vitro cultures because the success of tissue culture depends majorly on effective and efficient elimination of both

exogenous and endogenous pathogens (Buckley and Reed, 1994).The surfaces of living plants are naturally infested by microorganisms from the environment more especially while dealing with woody plants. Surface sterilization is usually done using chemical solutions whose concentrations and duration of exposure should be critically determined. In the present study different concentrations and exposure times of sodium hypochlorite and ethanol in combination with a caberndazim wash for 10 minutes were evaluated to determine their effectiveness for surface sterilization of wild blackberry species.

Furthermore, different explant sources respond differently. According to Chern*et al*., (1993), different explant sources have different growth potential due to differences in age, endogenous metabolic status and differential genome. Therefore different sources of explants were used in this study for wild blackberry species. In addition, the size of the explants determines the rate of survival of the explants. Long-sized stemsof nodal sections explants are most responsive*in vitro.* In this study, *Rubus apatelus* was difficult to respond and thus different sizes were sort for its micropropagation.

II. Materials And Methods

Experimental Site

The experiment was conducted at plant molecular crop research laboratory Department of crops, Horticulture and Soils, Egerton University.

Plant Selection

The shoots were obtained from the Horticultural teaching and reaserch from Rubus fruiticosus, Rubus apatelus and Rubus volkensis. The wild species were selected because; *Rubus apatelus* was the most vigorously in terms of growth and produces more fruits per season thus a potential choice for commercialization. *Rubus volkensis* had the highest branching rate and hence may give a best option for a root stock for blackberry species (Omondi*et al*., 2016). The species fruiticosus was selected because it has been adopted for commercialization in Kenya but yet a rapid propagation protocol has not been developed for the same.

Stock Solution and Media Preparation

Murashige and Skoog (MS) (Murashige and Skoog, 1962) media was prepared by dissolving the appropriate amount of macro and micro nutrients, and organic supplements in distilled water. Similarly, stock solutions of growth regulators were prepared at the ratio of 1mg plant hormone: 1ml double distilled water and stored in refrigerator at 6°C until use. The MS culture media was prepared from its respective stock solutions using the 30g/l sucrose. The pH of the culture medium was adjusted to 5.8 before autoclaving at 121°C for 20 minutes. 8g/l agar was used for solidification.

Sterilization of the Explants

The shoots were washed under running tap water for 30 min. Shoots were then cut into 1.0 cm pieces containing axillary winter buds. They were placed in sterile bottles and later used for different treatments as shown in the (table 1). To ensure that the disinfectant was in good contact with explant, tween 20 was added. After decontamination treatments, all treatments were rinsed three times with distilled sterile water. Nodal sections with axillary buds were isolated and inoculated in culture medium containing the MS macro nutrients (Murashige and Skoog, 1962),

Table 1. sterilization treatments

	Treatment
T1	**Wash with tap water** 5% NaOCl for 5 min 70% ethanol for 5 min
T2	**Tap water** 50% ethanol for 20 min 10% NaOCl for 10 min
T3	Tap water with tween 20 25% ethanol for 12 min 25% NaOCl for 12 min
T4	70% ethanol for 5 min 20% NaOCl for 5min
T5	20% NaOCl for 15 min
T6	25% Ethanol for 10 min 25% NaOCl for 10 min
T7	25% Ethanol for 5 min 25% NaOCl for 5 min
T8	Control – without sterilization
T9	Wash with tap water Caberndezim for 10 min5%

	NaOCl for 2 min 70% ethanol for 2 min

Effects of the part of explant used for micropropagation for wild blackberry species

Different parts of blackberry plants were evaluated nodal sections withNodal sections with mature stem sections nodal sections with apical stem sections, auxiliary buds without stem sections as shown in (table 2)

Table 2. Treatments for different plant part used for micropropagation of wild blackberry species

Source of explants
Nodal sections with mature stem sections
Nodal sections with apical stem sections
Auxiliary buds without stem sections

Effect of Explant Size on Survival rate of Rubus apatelus*in vitro*

The effect of explant size on axillary bud formation (proliferation) was evaluated by culturing explants of different sizes (0.7- 1.0cm, 1.1- 2.0 cm and 2.1- 3.0 cm of stems of nodal sections) on the proliferation medium. Each experiment included three replicates with at least five explants per replicate. For the proliferation experiments, the number of shoots that developed were recorded 1 month after the beginning of the experiment.

Table 3. Treatments for different sizes used for micropropation of *Rubus apatelus*

Explant size
0.7- 1.0cm of stems of nodal sections
1.1- 2.0 cm of stems of nodal sections
2.1- 3.0 cm of stems of nodal sections

Data Collection and Analysis

All experiments were laid in Complete Randomized Design composed of three replications per treatment. Each vessel consisted of eight explants. Data were collected on the following variables were evaluated: number of explants that survived per growing vessel, number of contaminated explants. Data were subjected to analysis of variance (ANOVA) using the general linear model procedure of the statistical analysis system (SAS) program (SAS institute I.nc, 2007). Significant means were separated using Tukey's honestly significant deference (Tukey's HSD) test ($P \leq 0.05$).

III. Results And Discussion

Effects of Different Sterilization Procedure on Contamination and Survival Rates of Wild Blackberry Species

There were significant effects ($p \leq 0.05$) of different sterilization procedures on the contamination and survival rates of wild blackberry species (table 4). Washing the plant materials with tap water followed by dipping them in Caberndezim for 10min, 5% NaOCl for 5 min then70% ethanol for 2 min resulted in the least contamination and the highest survival rates of 5.11 explants per vessel

Table 4: Means of percent survival and contaminations rates e of wild blackberry species in vitro

	Treatment	Contamination rates per culture vessel	Survival rates per culture vessel
T1	**Wash with tap water** 5% NaOCl for 5 min 70% ethanol for 5 min	9.72 ± 6.05^{e}	0.111 ± 0.719^{d}
T2	**Tap water** 50% ethanol for 20 min 10% NaOCl for 10 min	58.33 ± 6.05^{bc}	2.000 ± 0.719^{bcd}
T3	Tap water with tween 20 25% ethanol for 12 min 25% NaOCl for 12 min	8.33 ± 6.05^{e}	1.889 ± 0.719^{bcd}
T4	70% ethanol for 5 min 20% NaOCl for 5min	27.78 ± 6.05^{de}	1.7778 ± 0.719^{bcd}
T5	20% NaOCl for 15 min	29.17 ± 6.05^{de}	3.111 ± 0.719^{b}
T6	25% Ethanol for 10 min 25% NaOCl for 10 min	38.61 ± 6.05^{cd}	2.222 ± 0.719^{bc}
T7	25% Ethanol for 5 min 25% NaOCl for 5 min	75.00 ± 6.05^{ab}	2.556 ± 0.719^{b}
T8	Control – without sterilization	100.00 ± 6.05^{a}	0.333 ± 0.719^{cd}
T9	Wash with tap water Caberndezim for 10 min5% NaOCl for 2 min	11.11 ± 6.05^{de}	5.111 ± 0.719^{a}

	70% ethanol for 2 min		

Means followed by the same letter are not significantly different at p≤0.05 according to Tukey's HSD test. It is important to eliminate foreign contaminants such as bacteria and fungi prior to in vitro culture establishment; however, it is often difficult to acquire sterile plant material. It thus, becomes problematic while dealing with woody plant material (Niedz and Bausher, 2002). Basically, woody plants are grown in soil for a number of years under ambient climatic conditions and hence are normally infected heavily with microorganisms both endogenously and exogenously, which are often difficult to control in vitro (Ahmad et al., 2003). Carbendazim, is considered a broad-spectrum fungicide of the benzimidazole family. This family of fungicides binds to microtubules and interferes with cell division and transport (Park *et al*., 1997). The fungicide is systemic thus controls endogenous fungi. The treatment that was less contaminated (T9) gave the highest survival rate of 5.11 explants per culture vessel affirming the fact that pathogens compete for nutrients in vitro (Cassells, 2001). Ethanol and sodium hypochlorite at high concentrations and longer exposure time were effective for surface sterilization but did improve survival of explants.

Effects of Blackberry Species on Percentage Bud break and Survival *in vitro*

The percentage bud break at 21 days varied significantly different (p≤0.05) among the three species (table 5). The percentage bud break at 21 days was highest with *rubusfruiticosus* with 83.33% and lowest with *rubusapatelus* (Table 5). The number of explants that survived per culture vessel was also significantly different. Four, zero and two explants per vessel was observed with *rubusfruiticosus, rubusapatelus* and *rubusvolkensis* respectively after 8 weeks of culture.

Table 5: Means of percent bud break and survival rates of wild blackberry species *in vitro*

Species	% Bud break at 21 days	Survival per vessel after 8 weeks
Rubus fruiticosus	83.33^{a}	4.2963 ± 0.2397^{a}
Rubus apatelus	33.33^{b}	0.0000 ± 0.239^{c}
Rubus volkensis	61.11^{a}	2.0741 ± 0.2397^{b}

Means followed by the same letter are not significantly different at p≤0.05 according to Tukey's HSD testDuring our study, it was noted that the species *Rubus apatelus* was woodierand may have released a lot of exudates into the culture medium which likely affected bud break. Most woody tropical plants are prone to phenolics oxidation also called browning or blackening of the culture medium resulting to inactivation of growth in the cultures (Amhad*et al*., 2013). Cut surfaces of woody plants produces phenols which cause activation of oxidative enzymes such as polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and peroxidase (POP) which leads to browning and hence death of the explant (Litz and Vijayakumar, 1988). Phenolics also restrict nutrient availability by the explants resulting to their death (Cheema and Hussain, 2004). *Rubus apatelus* showed high fungual and bacterial contamination. Fungal contaminants increase culture mortality (Kane, 2003).

Effects of Explant Source on the Percentage Survival of Wild Blackberry Species *in vitro*

There was significant at p≤0.05 effect of the different blackberry explant sources and species on the percentage survival *in vitro*(table 6). Nodal sections with mature stem sections gave the highest percentage response of 66.667% in all the species. *Rubus fruiticosus* had the highest percentage survival of 56.944. Of the wild species *Rubus volkensis* had a higher response of 40.278% as compared to *Rubus apatelus* with 8.333%.

Table 6: Means of percent survival by explant source of wild blackberry species *in vitro*

Source of explants	% survival of wild blackberry
Nodal sections with mature stem sections	15.278 ± 2.23^{c}
Nodal sections with apical stem sections	66.667 ± 2.23^{a}
Auxiliary buds without stem sections	23.611 ± 2.23^{b}

Means followed by the same letter are not significantly different at p≤0.05 according to Tukey's HSD testNodal sections with apical stem sections showed highest survival rates due to less production of phenolic compound. Nodal sections with mature stem sections gave the lowest rate of survival due to production of a lot of phenolic compounds which led to oxidation of the medium resulting in availability of nutrients by the explant. Studies by (Bonga 1987, Sanchez and Vieitez 1991) showed that juvenile tissue can usually be more easily cultured in vitro than mature tissue explants. Nodal sections from mature branch parts explants showed poorer proliferation than nodal from explants, perhaps reflecting different physiological states of the buds at the different stem positions.

Effects of the Interaction between Explant Source and Blackberry Species on Percentage Response *in vitro*

There were significant at ($p \leq 0.05$) effects of explant source and species interaction on the percentage survival of wild blackberry plants(table 7). Nodal sections with apical stem sections gave the highest percentage response among all the three species with *Rubus fruiticosus*, *Rubus apatelus* and *Rubus volkensis* having 100%, 16% and 83% respectively.

Table 7: effects of part of explant and species on the percentage survival

Source of explant	species	% survival rates
Nodal sections from with mature stem sections	*Rubus fruiticosus*	29.167 ± 3.867^{d}
Nodal sections from with mature stem sections	*Rubus apatelus*	0.000 ± 3.867^{f}
Nodal sections from with mature stem sections	*Rubus volkensis*	16.667 ± 3.867^{e}
Nodal sections with apical stem sections	*Rubus fruiticosus*	100.000 ± 3.867^{a}
Nodal sections with apical stem sections	*Rubus apatelus*	16.667 ± 3.867^{e}
Nodal sections with apical stem sections	*Rubus volkensis*	83.333 ± 3.867^{b}
Auxiliary buds without stem sections	*Rubus fruiticosus*	41.667 ± 3.867^{c}
Auxiliary buds without stem sections	*Rubus apatelus*	08.333 ± 3.867^{ef}
Auxiliary buds without stem sections	*Rubus volkensis*	20.833 ± 3.867^{e}

Means followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey's HSD testAccording to the present findings, nodal sections with apical stem sections of the species *Rubus fruiticosus*gave 100% in vitro survival. Apical sections produce little phenolic exudates. This species may have survived best in vitro because it has undergone a lot of genetic and environmental modification.

Effects of Different Apical Nodal Section Sizes on the Percentage Response to Growth of *Rubus apatelus*

The species *Rubus apatelus* had significantly ($p \leq 0.05$) poor responses in percent survival rates and bud break in vitro. As a result different sizes experiments were conducted to determine the best size for micropropagation. The percentage response was highly significant at ($p \leq 0.05$) with the different sizes(table 8). Nodal sections cut with >2 cm stem cutting had the highest response of 93.33%. Less than 1 cm cuttings showed the lowest percentage response of 6.667%.

Table 8: Means of survival percent for different explant sizes for *Rubus apatelus in vitro*

Explant size	%survival
0.7- 1.0cm of stems of nodal sections	6.667^{c}
1.1- 2.0 cm of stems of nodal sections	26.667^{b}
2.1- 3.0 cm of stems of nodal sections	93.333^{a}

Means followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey's HSD testSize of explant significantly affected shoot regeneration. Long-sized (3cm) of stems of nodal sections explants were most responsive (93.33%). Decrease in size of the explant resulted in reduced regeneration response. In a report by Frary and Earle (1996) there is a reduction in shoot regeneration response with decreasing size of explants. High amounts of phenolic compounds that were being released to the culture medium led to the death of the small size explants. Phenolic secretions and other exudates in plant tissue culture systems lessen explant initiation, growth, and development which are more pronounced with small sized (Ozyigit, 2008). The large sizes gave high responses due to more supplies of carbohydrates.

Conclusions and recommendations

Mostly sterilization procedures for micropropagation are conducted with 70% ethanol and 1–3% NaOCl. In this paper, results showed that during the sterilization procedure an initial 10 minutes wash of the explant using carbandazim followed by 5% NaOCl then 70% ethanol showed also good results for the surface sterilization of 'fruiticosus', 'apatelus' and 'volkensis' nodiums. Sterilization procedures are different and depend on the tissue type and the nature of the explant used for micropropagation.

In addition, type of explants like petiole, hypocotyle, epicotylecotyledonary leaf, embryo, internode leaf and root explants significantly effect on plant tissue culture process. Our results showed that nodiums from apical sections responds best in vitro.

Further, different sizes of explants influences shoot proliferation. In this study we determined the appropriate explants size for high shoots proliferation. These findings could be a useful tool to improve the rate of in vitro multiplication of Rubus apatelus.

More studies on sterilization protocol, best source of explant and sizes best for micropropagation can be done for the other different wild blackberry species in Kenya.

Acknowledge

The authors would like to thank Regional Universities Forum for Capacity building in Agriculture (RUFORUM) for the financial support to this project.

References

[1]. Ahmed, M.F. 2002. Studies on the tissue culture and potential for the development of a genetic transformation system for avocados (*Perseaamericana* Mill.).Ph.D. Thesis, Univ. of Sydney, Australia.
[2]. Bonga, J.M. andAderkas P. (1992). In vitro culture of trees. Kluwer Academic Publishers, Dordrecht, *The Netherlands*, pp 72—125
[3]. Buckley, P.M. and Reed, B.M. (1994): Antibiotic susceptibility of plant associated bacteria. *Hort. Sci*.29:434.
[4]. Cassells, A.C. (2001). Contamination and its impact in tissue culture. *ActaHortic*. 560, 353-359 DOI: 10.17660/*Acta Hortic*.2001.560.66
[5]. Cheema K.L and Hussain M (2004).Micropropagation of sugarcane through apical bud and axillary bud.*International journal of agriculture and biology* 2: 257-259
[6]. Finn, C. E. and Clark, J. R. (2011). Emergence of blackberry as a world crop.*ChronicaHorticulturae*, 51(3), 13-18.
[7]. Fira, A.,Clapa, D. and Simu, M. (2014) Studies regarding the micropropagation of some blackberry cultivars. *Bulletin UASVM Horticulture*, v.71, p.29-37
[8]. https://doi.org/10.17660/ActaHortic.2001.560.66
Kane M (2003). Bacterial and fungal indexing of tissue cultures
[9]. http://www.hos.ufl.edu/moreweb/TissueCulture/class1/Bacterial%20and%20fungal%20indexing%20of20tissue%20cultures.doc
[10]. Litz R. E. and Vijayakumar (1988).In vitro somatic embryogenesis from the nucellus of Mango. ActaHortic 231: 473-475.
[11]. Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologiaplantarum*, 15(3), 473-497.
[12]. Niedz, R.P. and M.G. Bausher. 2002. Control of In vitro contamination of explants from green house and field grown trees. *In vitro Cell*. Dev. Biol. Plant, 38: 468-471
[13]. Odutayo, O.I., Oso, R.T., Akynyemi, B.O., Amusa, N.A. (2004): Microbial contaminants of cultured Hibiscus cannabinus and Telfaria occidentalis tissues. *Afr. J. Biotechnol*. 3(9):473-476.
[14]. Omondi, K.O., Gesimba, R.M. and Wolukau, J.N (2016).Performance of Wild Blackberry species in Kenya under conventional production. RUFORUM Working Document Series (ISSN 1607-9345) No. 14 (2): 627 - 632. Available from http://repository.ruforum.org
[15]. Park, S.Y., O.J. Jung, Y.R. Chung, and C.W. Lee. 1997. Isolation and characterization of a benomyl-resistant form of beta-tubulin-encoding gene from the phytopathogenic fungus *Botryotiniafuckeliana*. Mol. Cells 28:104-109.
[16]. Sanchez, M.C. and Vieitez A.M. (1991). In vitro morphogenic competence of basal sprouts and crown branches of mature chestnut. *Tree Physiology*. 8:59--70
[17]. Skirvin, R.M.; Chu, M.C. & Gomez, E. In vitro propagation of thornless trailing blackberries. *HortScience*, 16(3): 310-312, 1981.
[18]. Webster S, Mitchell S, Ahmad M (2003). A novel surface sterilization method for reducing microbial contamination of field grown medicinal explants intended for in vitro culture. Biotechnology Centre, UWI, Mona, Kingston. 7

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