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Vanda teres photo by SR Orquídeas

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The title of the article should be written in compact, clear, and informative sentence, preferably not more than 20 words. Name of author(s) should be completely written, especially for the first and the last name. **Name and institution** address should also be completely written with street name and number (location), postal code, telephone number, facsimile number, and email address. We choose local names in Bahasa Indonesia for universities in Indonesia. The mention of "strata" program, should be avoided. Manuscript written by a group, author for correspondence along with address is required (marked with "♥"). **The title page** (first page) should include title of the article, full name(s), institution(s) and address(es) of the author(s); the corresponding authors detailed postage and e-mail addresses (P), and phone (O) and fax numbers (O).

Abstract A concise abstract is required (about 200 words). The abstract should be informative and state briefly the aim of the research, the principal results and major conclusions. An abstract is often presented separately from the article, thus it must be able to stand alone (completely self-explanatory). References should not be cited, but if essential, then cite the author(s) and year(s). Abbreviations should be avoided, but if essential, they must be defined at their first mention. **Keywords** are about five words, covering scientific and local name (if any), research themes, and special methods used; and sorted from A to Z. **Abbreviations** (if any): All important abbreviations must be defined at their first mention there. **Running title** is about five words.

Introduction is about 600 words, covering the aims of the research and provide an adequate background, avoiding a detailed literature survey or a summary of the results. **Materials and Methods** should emphasize on the procedures and data analysis. **Results and Discussion** should be written as a series of connecting sentences, however, for a manuscript with long discussion should be divided into subtitles. Thorough discussion represents the causal effect mainly explains why and how the results of the research were taken place, and do not only re-express the mentioned results in the form of sentences. **Concluding** sentence should be given at the end of the discussion. **Acknowledgements** are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

Figures and Tables of a maximum of three pages should be clearly presented. The title of a picture is written down below the picture, while the title of a table is written above the table. Colored figures can only be accepted if the information in the manuscript can lose without those images; the chart is preferred to use black and white images. The author could consign any picture or photo for the front cover, although it does not print in the manuscript. All images property of others should be mentioned the source. Author is suggested referring to Wikipedia for international boundaries and Google Earth for satellite imagery. If not specifically mentioned, it is assumed to refer to these sources. **There is no appendix**, all data or data analysis is incorporated into Results and Discussions. For broad data, it can be displayed on the website as a supplement.

References Preferably 80% of it comes from scientific journals published in the last 10 years. In the text, give the author names followed by the year of publication and arrange from oldest to newest and from A to Z; in citing an article written by two authors, both of them should be mentioned; however, for three and more authors only the first author is mentioned followed by et al. For example, Saharjo and Nurhayati (2006) or (Boonkerd 2003a, b, c; Sugiyarto 2004; El-Bana and Nijs 2005; Balagadde et al. 2008; Webb et al. 2008). Extent citation should be avoided, as shown with the word "cit." Reference to unpublished data and personal communication should not appear in the list but should be cited in the text only (e.g., Rifai MA 2007, pers. com. (personal communication); Setyawan AD 2007, unpublished data). In the reference list, the references should be listed in alphabetical order. Names of journals should be abbreviated. Always use the standard abbreviation of a journal's name according to the **ISSN List of Title Word Abbreviations** (www.issn.org/2-22661-LTWA-online.php). Please include DOI links for journal papers. The following examples are for guidance.

Journal:

Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158. DOI: 10.13057/biodiv/d070213.

The usage of "et al." in long author lists will also be accepted:

Smith J. Jones M Jr. Houghton L et al. 1999. Future of health insurance. N Engl J Med 965: 325-329. DOI: 10.10007/s002149800025.

Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in the book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds.). Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.). Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from the internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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Review: Factors affecting mass propagation of *Vanda* orchid in vitro

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Abstract. *Setiaji A, Annisa RRR, Santoso AD, Kinasih A, Riyadi ADR. 2021. Review: Factors affecting mass propagation of Vanda orchid in vitro. Cell Biol Dev 5: 51-62.* For the past decade, *Vanda* has been the primadonna of ornamental orchids in the south and southeast Asia, along with *Phalaenopsis* and *Dendrobium*. Along with the increase in demand for *Vanda*, this genus has faced several threats, from illegal collection to habitat loss. Therefore, mass propagation through in vitro culture is a promising strategy to ensure sustainable business in horticulture and conservation. This review provides an overview and synthesizes various *Vanda* in vitro culture literature. We showed the researchers' preferences on several aspects for growing *Vanda*, including species, basal medium, plant growth regulators, explant, and culture conditions. The most commonly used as explants are seeds or protocorms, growing on Murashige & Skoog or Vacin & Went medium. This medium can be added banana homogenate to increase its nutritional value. *Vanda* seedlings can be incubated at 25 ± 1-3°C, with a lighting intensity of 50 µmol m^{-2} s^{-1} at 12/16 h PP. Choosing a medium that is cheaper but still rich in nutrients and its additives, especially during the subculture phase; selecting explants that are responsive and minimizing the possibility of contamination; as well as seeing the target market in particular, can make *Vanda*'s propagation efforts more effective, efficient, and profitable.

Keywords: Efficient protocols, in vitro culture, Orchidaceae, *Vanda*

Abbreviations: KC: Knudson's C medium (Knudson 1922); Mitra: Mitra et al. (1976) medium; MS: Murashige and Skoog (1962) medium; ND: New Dogashima medium (Tokuhara and Mii 1993); Nitsch: Nitsch and Nitsch (1969) medium; NP: New Phalaenopsis medium (Ichihashi 1992); P723: P723 Orchid Seed Sowing Medium (PhytoTechnology Laboratories, Inc.); RT: Raghavan and Torrey (1964) medium; SH: Schenk and Hildebrandt (1972) medium; VW: Vacin and Went (1949) medium; 2,4-D: 2,4-dichlorophenoxyacetic acid; 2-iP: N6-isopentenyladenine; BAP: 6-benzylaminopurine; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; Kn: kinetin; NAA: a-naphthaleneacetic acid; TDZ: thidiazuron; BP: banana pulp; CH: casein hydroxylate; CM: coconut milk; CW: coconut water; PP: potato pulp; TJ: tomato juice; YE: yeast extract; PVP: polyvinyl pyrrolidone; PLB: protocorm like bodies; RH: Relative Humidity; PP: photoperiod

INTRODUCTION

Orchidaceae is the second largest family of angiosperms after Asteraceae. There are many approximate versions of orchid species numbers because almost every expedition in biodiversity hotspots is reported to discover new species. A provisional checklist suggests 28,000 species of orchids, including 736 recognized genera, represent 8% of species of the angiosperms (Chase et al. 2015; Christenhusz and Byng 2016; Willis 2017). It exceeded the estimated number (25,000) (Atwood 1986).

The fascinating charisma of orchids defines them as ornamental plants in terms of color and the uniqueness of the flower shape. Orchid flowers could be kept indoors in fresh conditions for a long time as a symbol of beauty (Rahman et al. 2008). Cut flowers of the hybrids of *Mokara*, *Dendrobium*, and *Vanda* remain fresh for 7-30 days, while *Phalaenopsis* and *Cattleya* remain fresh for 1-4 weeks and 18-28 days for *Aranda* (De et al. 2014). In addition, orchid flowers have persistent perianth characters, unlike other cut flowers that easily fall off (Rahman et al. 2009). Today, orchid cultivation is an international business with great potential to participate in countries' economic growth. In the world floriculture trade, around 8% of sales are covered by orchids (Martin and Madassery 2006). In the ornamental plant industry, they are the second favorite cut flowers and potted plants (Hossain 2008).

One of the widely cultivated orchids in Southeast Asia and the Indian subcontinent is the genus *Vanda*, which was established by Sir W. Jones in 1795. His type species of this genus is *Vanda roxburghi*. *Vanda* is a monopodial orchid and mostly epiphytic (Islam et al. 2014). About 184 plant species are native to China, the Himalayas, Bangladesh, Indonesia, and northern Australia, of which 62 are accepted names, 122 are synonyms, and 5 remain unresolved (The Plant List 2019).

The name of *Vanda* came from an Indian language called Sanskrit (Garay 1974), which means that people like these plants by their fragrance, color, and flower shape. Many *Vanda* hybrids have characteristics preferred to mass

consumption, such as variable color pallets, fragrant flowers, free-blooming, long-lasting flowers, multiple inflorescences, compact growth habits, and cold tolerance. These superior traits make *Vanda* become great potential to dominate the American and European markets. In the 1950s, Hawaii, United States, became the center of *Vanda* orchid development, where they produced primary and secondary hybrids of *Vanda* with round and large-sized flowers. Later in the 1960s, Hawaii was replaced by Thailand. *Vanda* orchid breeders in Thailand produce more complex *Vanda* hybrids due to the segregation of progeny genes with new flower colors and shapes (Motes 2004).

One of the obstacles of *Vanda* cultivation is it requires three or more years of maintenance to reach flowering size since deflasking (compared to *Phalaenopsis*, which requires only 18–24 months). In addition, small-scale production and duration of *Vanda* culture cause the relatively high per-unit cost of production, which causes high selling prices at the farm level (Johnson and Kane 2007). Like some other orchid genera, Vanda was also threatened by habitat destruction, climate change, and unsustainable harvest (often illegal) for horticulture, food, or medicine (Fay 2018).

Mass production of orchids is important to meet the demand of orchid consumers and innovation for the world floriculture industry. There are many ways to propagate orchids. The conventional propagation was the separation of pseudobulb clumps and keiki. Still, these methods are unsuitable for mass production because they are inefficient in time and space and have high risks of the parent plant's death. Tissue culture is now an established effective propagation method, offers large-scale productions, and ensures clonal stability, irrespective of season and weather (Singh and Duggal 2009; Teixeira da Silva et al. 2015). Knudson (1922) developed a protocol for asymbiotic in vitro orchid seed germination on a medium containing mineral nutrients and sugar. The first experiments on *Vanda* in vitro culture were carried out at the University of Singapore with callus derived from seedlings in undefined media containing tomato juice and 2,4-Dichlorophenoxy acetic acid (2,4-D) (Rao 1963; Rao 1967). This technique continues to be developed, including applied along with genetic engineering, and becomes an important method for mass-scale propagation and conservation of orchid species. The main objective of this review is to provide a thorough understanding of *Vanda's* germplasm response to in vitro conditions by compiling what is known from various published literature and research. Data representation in the chart is based on the proportion of the number of times a study is to the total number of outcomes. Detailed information is shown in Table S1.

IN VITRO PROPAGATION FOR CONSERVATION AND SUSTAINABLE UTILIZATION

Vanda is important in the ecosystem, especially related to the host plant and its symbiotic microorganisms. Its aerial roots can absorb and retain moisture, attracting various bacteria and fungi for symbiosis, beginning with seed germination. These microorganisms provide nutrients and increase resistance against pathogens through induced systemic response (ISR) mechanisms and metabolites excreted by these microbes (Pieterse et al. 2014). These microorganisms, which are generally bacteria, known as plant growth-promoting rhizospheric bacteria (PGPR), can be isolated and applied to other plants as biocontrol agents and biostimulants (Glick 2015). *Vanda*'s ethnobotany is about aesthetics and promises the exploration of various compounds for health benefits.

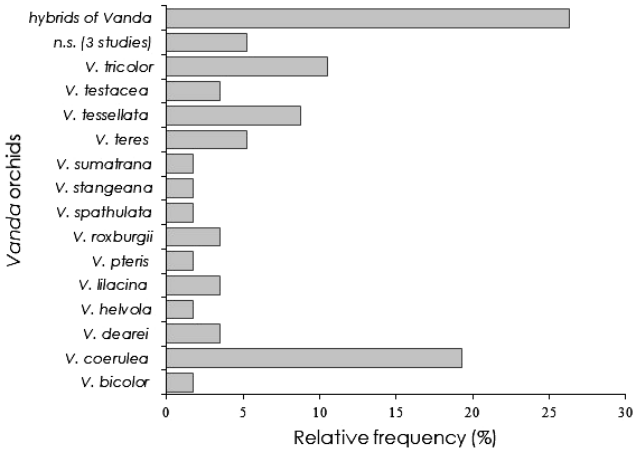

Figure 1. Micropropagation studies' relative frequency (%) shows various *Vanda* species and hybrids. Some of the studies use more than one type of orchid. n.s. not specified.

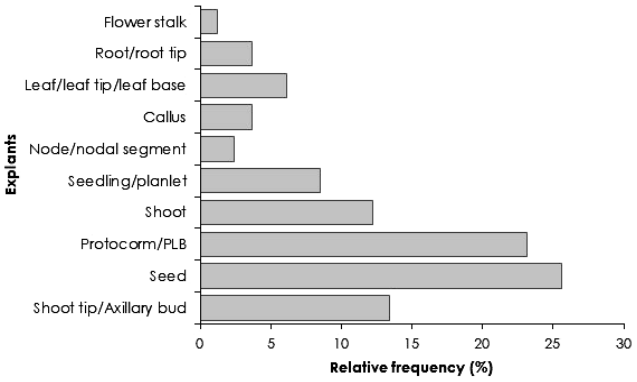

Figure 2. The relative frequency (%) of micropropagation substudies shows different explants used in *Vanda* in vitro culture. Some studies used more than one explants.

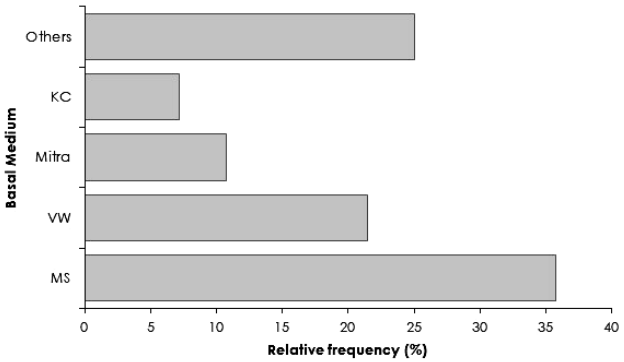

Figure 3. Relative frequency (%) of micropropagation substudies showing basal medium used in *Vanda* in vitro culture. Some of the studies used more than one basal medium

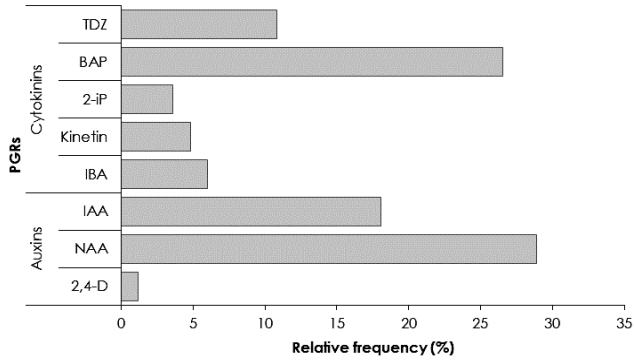

Figure 6. The relative frequency (%) of micropropagation substudies shows different plant growth regulators (PGRs) used in *Vanda* in vitro culture. Some of the studies may use more than one substudies

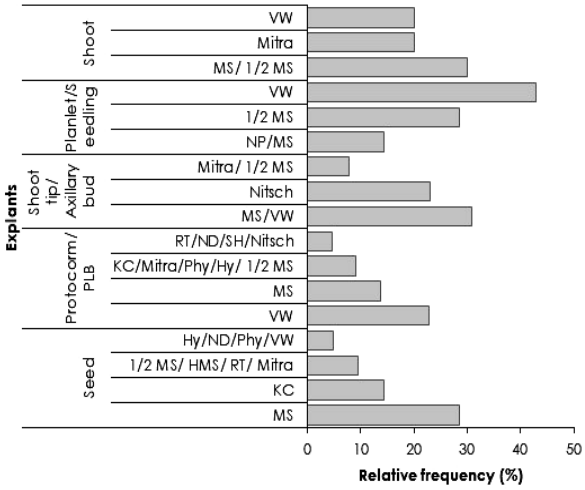

Figure 4. Relative frequency (%) of micropropagation studies showing basal medium used based on explant types in *Vanda* in vitro culture. A study may have one or more types of explant

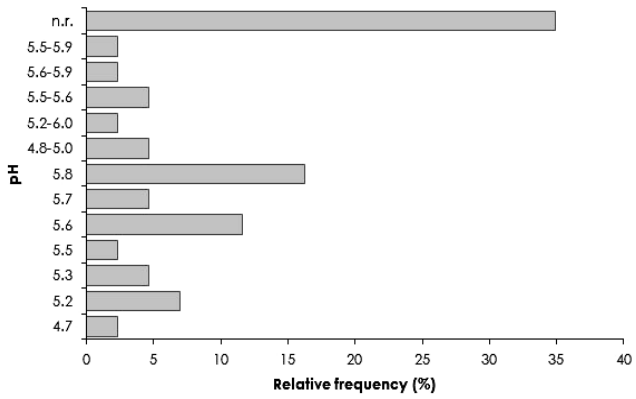

Figure 5. Relative frequency (%) of micropropagation studies showing different pHs used of the basal medium in *Vanda* in vitro culture. n.r. not reported

In addition to medicinal uses, especially in India, Nepal, China, and Bangladesh, Khan et al. (2019) have summarized the use of *Vanda* in traditional medicine and its bioactive compounds. The class of compounds detected included eucomic acid and its derivatives from *V. teres* (Simmler et al. 2011); Phenanthrene derivatives from *V. tessellata*, *V. parviflora*, and *V. coerulea* (Anuradha et al. 2008; Anuradha and Rao, 1998; Simmler et al. 2010); Bibenzyl derivatives from *V. coerulea* and *V. roxhburghii* (Simmler et al. 2010; Uddin et al. 2015); Phenolic compounds from *V. roxburghii*, *V. parishii*, and *V. tessellata* (Chawla et al. 1992; Dahmén and Leander 1976; Prakash and Bais 2016); Anthocyanins from *V.* hybrid (*V. teres* x *V. hookeriana*) (Junka et al. 2012); Alkaloids from *V. hindsii* (Brandange and Granelli 1973); Steroids and triterpenoids from *V. roxburghii* (Mohammed-Usman et al. 2012). Based on the examination of *Vanda* extracts of various species, these orchids are known to have pharmacological activities like anti-inflammatory, antioxidant neuroprotective, membrane stabilizing, anti-aging, hepatoprotective, antimicrobial, and wound healing activities (Khan et al. 2019). Traditional uses usually treat rheumatism, dyspepsia, indigestion, piles, wounds, bronchitis, and hepatitis (Khan et al. 2019). However, more research is needed to select unique compounds with strong bioactivity potential. A further prospect is the extraction of materials with whole plants that need to be replaced with plant materials from in vitro cultures, such as callus cultures and cell suspensions. In vitro culture can provide optimal conditions for synthesizing these compounds with additional precursors, and the amount of the yield can be standardized (Setiaji et al. 2020).

Due to the limited population size and providing expected properties in medicine, and the rise of vulnerability in the future, conservation efforts are important. The reintroduction of *Vanda* is mostly undertaken by returning plants taken from nature and donations from certain nurseries. Mass breeding through tissue culture promises to speed up the reintroduction and translocation of orchids. However, these two processes are important, and conservation programs' primary goals

should include maintaining, managing, and restoring habitats that support orchid populations. In vitro propagation studies provide a starting point for conservation efforts. Unfortunately, research publications about *Vanda* have not been as much as *Dendrobium*, *Phalaenopsis*, *Oncidium*, *Cattleya*, and another popular genus of orchids that may have reached hundreds. Still, this information could be used as initial information to develop in vitro *Vanda* cultivation.

Vanda breeding, both with genetic transformation and interspecific hybrids, is the consumer's favorite since they have various choices in flower colors and other superior properties. On the other hand, wild *Vanda* tends to have some disadvantages, such as difficulty adjusting the growth to the local climate, usually does not meet the dosage of commercial fertilizers and hormones (maintainers need to determine the optimal dose for the orchid by themselves), and more expensive. So far, the relative frequency of research conducting on *Vanda* hybrid micropropagation is 26.3%.

Based on the number of papers that have been published, species of *V. coerulea* (19.3%), *V. tricolor* (10.6%), and *V. tesselata* (8.8%) are the most widely studied (Figure 1). *V. coerulea* is one of the most popular native orchids found in the northeastern region of India, with a range of distribution extending to China (southern Yunnan). The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) listed this species in appendix 2 (removed from appendix 1 due to the discovery of new populations, especially in the Himalayas region (Christophe 2012) and the global conservation status is vulnerable (Walter and Gillet 1998). Habitat loss and degradation, mainly from human activities and illegal hunting, were the major cause of the decline in the population of these orchids. In other regions, *V. tricolor* (appendix 2) also faces the same threats. It is widespread and highly cultivated in South East Asia, while the wild populations are small and highly fragmented, especially in Java and Bali (Gardiner 2007). Gardiner (2007) reported that this species has been rare in nature due to over-collecting and natural disasters such as Mount Merapi eruption, one of the most active volcanoes in Indonesia with a 4-year eruption cycle. Anticipating a similar threat, the researchers developed an in vitro propagation technique for *V. tesselata* earlier to maintain a population whose trend tended to decline even though it still had the least concern status (Khela and Chadburn 2014). The distribution of V. tesselata is broad enough to cover Bangladesh, India, Myanmar, Nepal, Sri Lanka, and Thailand.

A successful campaign of *Vanda* conservation efforts has been carried out by Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), India. The successful restoration of the *Smithsonia maculata* orchid, carried out by JNTBGRI in 1993, was expanded to other species, including *Vanda coerulea*, *Vanda wightii*, and *Vanda thwaitesii*. The success of in vitro propagation which can provide mass and quality of plants, has an important role in the success of this conservation program. Its reintroduction success reaches 80-95% after establishment for 1-2 years (Rajasekharan and Wani 2020). An integrated conservation program involving various professionals in ecology, pollination biology, tissue culture, microbiology, and genetic populations has proven effective in planning and implementing real conservation efforts.

EXPLANT, CULTURE MEDIUM AND ITS CONSTITUENTS

Explants used in *in vitro* propagation

Explant selections are an important factor to consider before initiating a culture method. Explants taken from potted plants in the greenhouse (ex vitro) may carry fungal and bacterial infections due to exposure to open environments. In monopodial orchids, such as *Vanda*, choosing the shoot tip as an explant could be caused death to the mother plant since monopodial orchid relies their growth on their apical dominance. Furthermore, a flower stalk could only be obtained during the flowering season (the flowering could be induced but cannot be continuously carried out). Indeed, the flower stalk of *Vanda* has limited plantable parts; for comparison, they have a shorter length than the stalk of *Phalaenopsis*, which is commonly used as an explant for *Phalaenopsis* micropropagation. The flower stalks of *Vanda* also mature rapidly, whereas young flower stalks are known better to use as explants. In general, choosing juveniles and other young tissues over mature parts needs to be considered. Other than that, the flowering plants of *Vanda* are 2-3 times more expensive than their vegetative plants.

Seeds are the most commonly used explant for *Vanda* propagation (25.6%) (Figure 2). The next preference explants used are protocorm (23.2%), shoot/ axillary tip (13.4%), shoot (12.2%), and others below 10% (seedling, nodal segment, callus, leaf, root, and flower stalk). Seeds could provide large quantities of explants where adult orchids plant are limited. Seeds could germinate even using a basal medium without adding hormones or complex organic matter, only. Seeds will grow into protocorms and become seedlings later (Yildiz 2012). Protocorms have the flexibility to induce shoots and roots and/or reproduce secondary protocorms/ PLBs (Sujjaritthurakarn and Kanchanapoom 2011; Setiaji et al. 2018). The protocorm phase usually begins when the bipolar structure cannot be distinguished between basal and apical (Setiari et al. 2016). By definition, protocorms are produced by seeds, whereas protocorm-like bodies (PLB) are produced by explants (Lee et al. 2013).

Seed culture is probably the most effective technique so far to get lots of new seedlings, despite the long maturity time of *Vanda* fruit capsules, which could reach 6-9 months or even up to 20 months (PhytoTech Labs 2019). This problem could be overcome by applying 6-Benzylaminopurine (BAP) and gibberellic acid (GA) hormones to stimulate flowering in plants, continued by spraying 6-30-30 sodium-phosphate-potassium fertilizer after pollination for fruit ripening. However, unripe seeds can still be planted and show better results in some cases. When the seeds are ripe, the inner coat surrounding the embryo may be thickened, making it difficult for water and

nutrients to reach the seeds. In addition, some seeds may carry some poor traits that lead to nonuniformity clones. That could be avoided by choosing superior breeds and maintaining their genetic content stability. The optimum preference for explants, medium, and incubation conditions will be explained later.

Culture media

In general, the most commonly used basal medium for *Vanda* cultures is MS (35.7%) and VW (Vacin and Went, 1949) (21.4%) (Figure 3). On the other hand, 28.5% of the sub-studies using seeds were planted on Murashige and Skoog medium (1962) (Figure 4). MS is widely used in a variety of plants, including orchids. This medium contains high concentrations of ammonia, potassium, and nitrates; and is relatively cheaper than other mediums, such as the White medium (Stewart Jr 2016). On the other hand, VW media was specifically intended for orchid species at the beginning of the formulation, especially for *Cymbidium*. In this medium, $Ca_3(PO_4)_2$ is added in abundant quantities, providing phosphate to increase the formation of PLB (protocorm-like bodies) (Teixeira da Silva 2012). The seeds of 18 different orchid genera, planted on the VW medium, produce a chance of more than 70% of protocorms formation (Kartikaningrum et al. 2017).

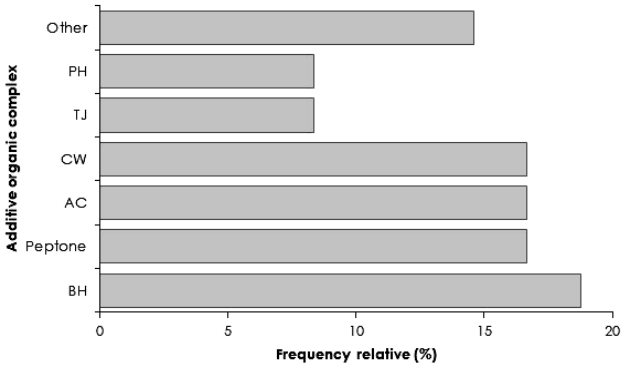

Figure 7. The relative frequency (%) of micropropagation substudies shows various additives used in *Vanda* in vitro culture. A study may have one or more substudies. PP potato pulp, TJ tomato juice, CW coconut water, AC activated charcoal, BP banana pulp

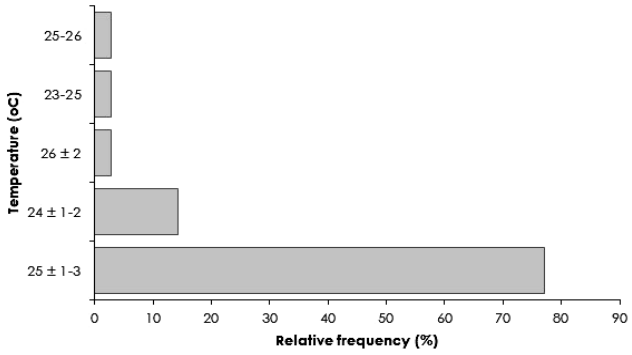

Figure 8. Relative frequency (%) of micropropagation studies showing different temperature conditions in *Vanda* in vitro culture

Table 1. Number of micropropagation studies showing different lighting conditions during the *Vanda* in vitro phase

		Photoperiod (h)				
		0	**12**	**16**	**24**	**nr**
Light intensity (µmol m^{-2} s^{-1})	25	-	-	1	-	
	30	-	-	2	-	1
	35	-	1	1	-	-
	40	-	1	1	-	-
	45	-	-	2	-	-
	50	-	4	3	-	-
	NR	1	-	2	1	-
	28-35	-	-	1	-	-
	30-50	-	1	-	-	-
	NR	4	-	-	-	-
	20-50	-	1	-	3	-
	37	-	-	1	-	-
	10	-	1	-	1	1
	9	-	-	-	2	-
	20-30	-	-	1	-	-
	56	-	-	1	-	-
	15	-	-	1	-	-
	100	-	-	-	-	1

The pH set for *Vanda* culture varies from 4.7-5.9 (Figure 5). The greatest preference was at pH 5.8 (16.3%) and after that, 5.6 and 5.2. The pH of the MS medium is usually set between 5.6-6.3. Adjusting the pH of the culture medium is important to ensure the plant's physiological processes are not disturbed. Acidic medium prevents the uptake of phosphoric acid, Ca^{2+}, and Mg^{2+}; alkalic medium prevents the uptake of iron, Cu^{2+}, Zn^{2+}, Mn^{2+}, and boron (Bell et al. 2020; Jakobsone and Osvalde 2019; Ichinose et al. 2018). In addition, pH affects the solubility and absorption of nutrients by activating certain enzymes and solidifying gelling agents; and preventing the absorption of toxic substances (Sahu et al. 2017; Lager et al. 2010). A slightly acid medium seems to be preferred by most orchids and is important for auxin action (Sarkar et al. 2009). Sachin (2015) reported that the highest protocorm formation on *V. tesselata* was observed at a temperature of 20°C and pH 5.5. It is difficult to determine whether the pH of the medium could affect the orchid seedlings because it is related to other culture media components.

Plant growth regulators

Plant growth regulators (PGRs) can be used simultaneously to match *Vanda* growth stages. The most commonly used PGRs in *Vanda* cultures, either as combined or in a single dose, are the cytokinins (6-Benzylaminopurine (BAP), kinetin (Kin), N6-isopentenyladenine (2-iP), and thidiazuron (TDZ); and auxin (indole-3-acetic acid (IAA)), indole- 3-butyric acid (IBA), 2,4-Dichlorophenoxyacetic acid (2,4-D), and a-naphthaleneacetic acid (NAA) (Fig. 6). In combination, 15.47% used higher concentrations of cytokinins such as BAP (4.44-66.6 µM), while 10.71% used a higher concentration of auxin such as NAA (0.27-8.06 µM). Single auxin (15.47%) is generally used to induce roots or germination with optimum concentrations ranging from

0.54-22.80 µM for NAA, while single cytokinin (10.71%) is generally used to induce shoots with optimum concentrations range of 0.91-11.35 µM for TDZ. The rest 41.66%, do not use any PGRs, and generally prefer to add complex organic materials for germination or seedling maintenance.

Organic complex sources

Organic complex materials contain different sucrose, fructose, agar, peptone, nicotinic acid, biotin, folic acid, auxin, glutamic acid, glycine, adenine, niacin, and nitrogen levels (Park and Yeung 2018; Acemi and Ozen 2019). Any of these components are responsible for promoting the growth and development of the cultures (Islam et al. 2015). The most commonly used complex organic materials in *Vanda* cultures are banana homogenate (18.8%), peptone (16.7%), activated charcoal (16.7%), coconut water (16.7%), tomato juice (8.3%), and potato homogenate (8.3%) (Figure 7). In *Vanda*, few papers explain the function of adding these additives because they may have complex effects and focus more on the effects of PGRs. However, the beneficial effects of complex organic materials (BH, CW, peptone) on the growth and differentiation of protocorms and seedlings have been carried out by Arditti (1979).

Banana is rich in carbohydrates, certain vitamins, minerals, carotenoids, and polyphenols. Usually, Studies that employed BH used a concentration of 3.5-15% (v/v) combined with auxin and/or auxin-cytokinin and other additives. BH might help stabilize the pH of the medium, which may change due to activated charcoal. The pH of the medium could drop due to the acid residues of HCl in AC since AC needs to be washed by HCl solutions in its production (George et al. 2008). Peptone generally consists of high tryptophan, a low molecular weight protein, vitamins, and plant growth factors. These factors may induce changes in *Vanda*, which can give plant cells an easily absorbed nitrogen source (George et al. 2008). CW can induce cell division, thus promoting early protocorm differentiation and a wide spectrum of growth factors, and has been successfully used in some orchid production (Intuwong and Sagawa 1973; Pyati et al. 2002). In epiphytic orchids, the addition of 15% CW to the basal medium can increase growth performance in various parameters: shoot length, number of roots, leaf width, leaf area, fresh and dry weight of shoots and roots, and stimulating new shoots (Baque et al. 2011; Yong et al. 2009; Paris et al. 2019). The main hormone contained in CW is IAA, while cytokinin, gibberellin, and abscisic acid are also detected (Yong et al. 2009; Tan et al. 2014).

The addition of activated charcoal improves the growth of *Vanda*. Some of the positive effects of AC are improved aeration, established polarity of microelements, stabilized substrate temperature, and adsorbs toxic substances (phenolic compounds), all because of the nature of AC which has small pores and a large surface area (Thomas 2008; Zeng et al. 2015). In addition, AC is suitable for root induction because it creates dark conditions of the medium in accordance with the underground root's original environment. AC and BAP can increase flowering frequency from 65% to 100%, increase in vitro germination and plantlet development, increase rhizome production and fresh weight gain during micropropagation, and increase the formation of orchid buds and promote bud induction of orchid seeds effectively (Thomas 2008). However, in some cases, adding 1% activated charcoal to culture media caused acidification, largely due to an increase in the hydrolysis of sucrose during sterilization (Saad and Elshahed 2012). Another disadvantage of AC is the adsorption is not selective; some beneficial substances may also be adsorbed.

INCUBATION CONDITION

Lighting, temperature, and humidity are important aspects for maintaining the incubation chamber to support plant growth and adapt to in vivo environments. However, this review does not explain the humidity conditions because too few papers have mentioned it since measuring humidity inside culture bottles may be difficult.

The studies on *Vanda* in vitro culture used the temperature ranging from 23-26ºC, but 25 ± 1-3ºC (77.1%) was most commonly used. The light intensity varies from 25 to 100 µmol m^{-2} s^{-1}, but the most widely used is 50 µmol m^{-2} s^{-1} at 12/16 h PP (7 studies). Only 5 studies report using dark culture; the light intensity is not reported (Tab. 1). All studies employ light-emitting diodes.

In conclusion, the growth of *Vanda* orchids during the in vitro phase requires optimal controlled conditions. It ensures seedlings' viability during acclimatization and uniformity during flowering induction in the greenhouse. This review attempts to infer the basic needs for in vitro culture in *Vanda* based on the preferences of previous studies. The most commonly used source of explants is seeds or protocorms planted on MS or VW medium with a pH of 5.8. Banana homogenate 3.5-15% is the most used additive. *Vanda* seedlings were mostly incubated at 25 ± 1-3ºC, with a lighting intensity of 50 µmol m^{-2} s^{-1} at 12/16 h PP. *Vanda's* in vitro culture technique still needs to be developed and expanded with the application of molecular biotechnology. The potential and uniqueness of ornamental, horticultural, and medicinal values are also slightly mentioned. This review can temporarily serve as a basis for *Vanda* producers to avoid confusion in choosing culture procedures from the various studies conducted.

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Table S1: Remarks on micropropagation of *Vanda* species and/or hybrids

Authors	Media	pH	Explant	PGR (µM)	Outcome	Additives	Incubation conditions	Spesies and/or hybrids
Aini et al. (2015)	MS	n.r.	Shoot tip	6.66 BA	PLB			*Vanda sumatrana*
Puspasari et al. (2018	NP	5.2-6.0	Seed		development of protocorm to seedling	0.1% P		*Vanda tricolor*
David et al. (2015)	KC	n.r.	Seed		development of protocorm to seedling	0.1% P	24-h PP, 25 ± 2 °C	*Vanda helvola*
	KC		Seed		Germination	10 or 15% TJ		
Begum et al. (2002)	MS	5.8	Axilary bud		Protocorm		12-h PP, 50 µmol m^{-2} s^{-1}, 26 ± 2°C, 78% RH	*Vanda pteris*
	MS		Protocorm	8.88 BA + 5.37 NAA	Shoot			
	1/2 MS		Shoot	9.84 IBA	Shoot produce root	0.1-0.2% AC + 15% BP along + 15% PP + 15% CM		
Bembecha et al. (2016)	1/2 MS	5.6-5.9	Seed		Germination		16-h PP, 28–35 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda stangeana*
	1/2 MS		Protocorm	2.7 NAA + 2.3 Kin	Protocorm multiplication			
	1/2 MS		Seedling		Planlet	3% BP		
	1/2 MS		Micropropagated shoots	5.7 NAA	Shoot produce root			
Bhattacharjee and Islam (2014)	MS	5.6-5.8	Shoot segment	5.37 NAA + 4.44 BA	Multiple shoot		16-h PP, 25 ± 2 ºC	*Vanda tessellata*
	MS		Seed		Germination			
	1/2 MS		Shoot	5.71 IAA	Shoot produce root			
Malabadi et al. (2004)	VW	5.8	Shoot tip	11.35 TDZ	PLB	0.2% CH + 0.05% L-glutamine + 0.025% P	100 µmol m^{-2} s^{-1}, 25 ± 2 ºC, 55-60% RH	*Vanda* coerulea
	VW		Shoot tip	11.42 IAA / 14.76 IBA / 16.11 NAA	Shoot produce root	0.2% CH + 0.05% L-glutamine + 0.025% P		
Deb et al. (2018)	MS	n.r.	Seed	3 NAA + 3 BA	PLB, planlet		12-h PP, 40 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda* bicolor
Decruse et al. (2003)	Mitra	5.6	Node	44.4 BA + 17.1 or 28.5 IAA and 66.6 BA + 28.5 or 40.0 IAA	Shoot/node		12-h PP, 30–50 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda spathulata*
	Mitra		Shoot	5.7 IAA	Shoot produce root	7.5% BP		
Dwiyani et al. (2015)	NP	n.r.	Seed		development of protocorm to seedling	10-20% TJ		*Vanda tricolor* var. *suavis*
	NP		Seedling		Planlet	10-20% TJ		
Gnasekaran et al. (2012)	VW	4.8-5.0	PLB		Secondary PLB	20% TJ + 10% CW	16-h PP, 30 µmol m^{-2} s^{-1}, 25 ± 1 ºC	*Vanda* Kasem's Delight
Hardjo and Savitri (2016)	1/2 MS	n.r.	Callus	0.27 NAA + 0.04 BA	Embryogenic callus		30 µmol m^{-2} s^{-1}, 24 ± 1 ºC	*Vanda tricolor* var. *pallida*
Hrahsel and Thangjam (2015)	MS	5.8	Seed		PLB		16-h PP, 35 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda coerulea*
	MS		PLB	22.80 IAA	shoot			
	MS		Shoot		Shoot produce root	0.075% banana extract		
Islam et al. (2011)	Hyponex	5.6	seed		Germination	20% PP	16-h PP, 45 µmol m^{-2} s^{-1}, 25 ± 1 ºC	*Vanda* roxburgii
	Hyponex		Protocorm		Planlet	10% PP		
Islam et al. (2014)	MS	n.r.	Seed	0.54 NAA	Germination	15% CW	16-h PP, 25 ± 1 ºC	*Vanda roxburghii*

Jawan et al. (2014)	1/4 Mitra, 1% sucrose	n.r.	Calli induced from leaf segment	4.54 TDZ + 0.54 NAA.	Callus maintain		24-h in the dark, 25±2°C	*Vanda dearei*
Jawan et al. (2010)	1/2 MS	5.3	Seeds germination		Protocorm	0.2% YE	24-h PP, 20-50 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda dearei*
Jitsopakul et al. (2013)	VW, 1% sucrose	5.2	Shoot tip	4.44 BA	Shoot and root		16-h PP, 37 µmol m^{-2} s^{-1}, 25 ± 3 ºC	*Vanda coerulea*
	VW, 1% sucrose		Seedling	2.69 NAA + 9.08 TDZ	Root multiplication			
Jhonson and Kane (2007)	½ MS	5.7	Seed		Germination		12-h PP, 50 µmol m^{-2} s^{-1}, 23 ± 2 ºC	*Vanda* Paki · (*Vanda* tessellata · *Vanda* cristata)
	½ MS		Seed		Germination		16-h PP, 50 µmol m^{-2} s^{-1}, 23 ± 2 ºC	(*Vanda* Joan Warne · *Vanda* Paki) · *Vanda* Loke
	P723		Seed		Germination		8 or 16-h PP, 50 µmol m^{-2} s^{-1}, 23 ± 2 ºC	*Vanda* Motes Primrose · Ascocenda Tavivat
David et al. (2008)	KC	n.r.	Protocorm	8.88 BA + 2.69 NAA	PLB		24-h PP, 20-50 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda helvola*
Jualang et al. (2014)	KC, 1% sucrose	5.3	Seed		Germination	0.5% YE	24-h PP, 20-50 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda dearei*
	KC		Protocorm		Seedling	20% CW		
Karyanti (2017)	MS	n.r.	Planlet	2.32 Kin	Shoot induction		10 µmol m^{-2} s^{-1}, 25-26 ºC	*Vanda douglas*
	MS		Planlet	2.27 TDZ	Shoot and leaf			
Kaur and Bhutani (2009)	Mitra	5.7	Foliar	4.44 BA alone/with 5.37 NAA	PLB proliferations and plantlet development	2% AC	12-h PP, 35 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda testacea*
Lang and Hang (2006)	MS	5.2	Root	13.62 TDZ + 13.63 2,4-D	Callus			*Vanda coerulea*
	MS		Callus derived stem	0.54 NAA + 13.62 TDZ	Somatic embryogenesis			
Manners et al. (2010)	MS	5.8 ± 0.02	Root	30 BA + 15 IAA	PLB		12-h PP, 50 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda coerulea*
Manners et al. (2011)	MS	5.8 ±	Seed	5 BA or 5 IAA	Germination		12-h PP, 50 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda coerulea*
	MS	0.02	Protocorm	5 BA + 15 IAA	Seedling			
Mathews and Rao, 1980)	RT or Mitra	5.5	Protocorm		Planlet		12-h PP, 10 µmol m^{-2} s^{-1}	*Vanda* TMA (*Vanda* Josephine Van Brero X *Vanda sanderiana*),
	RT or Mitra		Seed		Germination			*Vanda* TMA × *Vanda roxburghii*
	RT or Mitra		Seed		Germination			*Vanda* TMA × *Vanda* Miss Joaquim
Mathews and Rao, 1985)	Nitsch	5.5-5.6	Protocorm		Protocorm differentiation	10-15% CM	24-h PP, 9 µmol m^{-2} s^{-1}, 25 ± 2 ºC, 55-60% RH	*Vanda* TMA × *Vanda* Miss Joaquim
Mathews and Roy, 1985)	Nitsch	5.5-5.6	Leaf base	5.71 IAA + 9.84 2iP	PLB		24-h PP, 9 µmol m^{-2} s^{-1}, 25 ± 2 ºC, 55-60% RH	
			Shoot tip	4.92 2iP + 5.71 IAA	protocorm			V TMA x V Miss Joaquim
			Shoot tip	4.92 2iP	protocorm			V TMA x V Miss Joaquim

			Shoot tip	5.71 IAA	green nodular protuberances			V TMA x V Miss Joaquim
Jain and Ochatt (2010)	VW	5.6	Nodal segment	9.29 Kin + 2.69 NAA	PLB	0.2% peptone + 0.1% AC	16-h PP, 50 µmol m^{-2} s^{-1}, 24 ± 2 ºC	*Vanda* teres
	VW		PLB		Shoot	15% CW + 0.2% peptone + 1.03 mM l-glutamine + 0.1% AC		
	VW		Shoot	4.92 IBA	Planlet	15% CW + 0.2% peptone + 0.1% AC, 5% BP		
Obsuwan and Thepsithar (2014)	VW	4.8-5.0	Planlet		Planlet maintain	10% 'Gros Michel' BP	16-h PP, 30 µmol m^{-2} s^{-1}, 25 ± 1 ºC	*Vanda* Tokyo Blue
	VW		Planlet		Planlet maintain	15% CW		
Pimda and Bunnag (2010)	ND	n.r.	Seed		Protocorm to planlet	1% PP	16-h PP, 40 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda lilacina*
	ND		Protocorm	13.32 BA	Protocorm proliferation			
Prakash et al. (2012)	MS	5.5-5.9	Seed		Germination and protocorm formation		16-h PP, 25 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda tessellata*
Rahman et al. (2009)	MS	5.8	Shoot tip	8.06 NAA + 4.44 BA	Shoot formation		16-h PP (20-30 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda tessellata*
	MS		Shoot	2.69 NAA + 4.92 IBA	Root formation			
Rineksane and Sukarjan (2015)	ND	n.r.	*Ex vitro* leaf	4.44 BA + 0.54 NAA or 8.88 BA + 0.54 NAA	Callus		24-h PP, 10 µmol m^{-2} s^{-1}, 23-25 ºC	*Vanda tricolor*
	ND		*In vitro* leaf	2.27 TDZ	Callus			
Roy et al. (2011)	Phytamax	5.6	Seed		Protocorm development		16-h PP, 56 µmol m^{-2} s^{-1}, 24 ± 2 ºC	*Vanda coerulea*
	Phytamax		Protocorm	5.36 NAA + 3.80 BA	PLB			
	Phytamax		PLB		Planlet	0.3% AC		
Sebastinraj et al. (2014)	1/2 MS	5.6	Seed		Protocorm		16-h PP, 15 µmol m^{-2} s^{-1}, 25 ± 2 ºC	*Vanda testacea*
				0.91 TDZ	Shoot			
				0.98 IBA	Root			
Seeni and Latha (2000)	Mitra	5.2	Proliferating buds / PLB	1.08 NAA	new bud or PLB, rapid growth of buds into shoot and emergence of shoot	3.5% BP + 30% CW	12-h PP (20-50 µmol m^{-2} s^{-1}, 24 ± 2 ºC	*Vanda coerulea*
	Mitra		Shoot	1.08 NAA	root	3.5% BP		
Sinha and Roy (2004)	VW	5.8	Seed	4.44 BA + 2.69 NAA	Germination	0.2% P	16-h PP, 50 µmol m^{-2} s^{-1}, 24 ± 1 ºC	*Vanda teres*
	VW		Protocorm	4.44 BA + 2.69 NAA	Shoot	0.2% P + 10% CW		
	VW		Shoot		Shoot multiplication	0.2% banana powder + 100 mg L^{-1} CH		
	1/2 MS		Planlet		Planlet maintain			
Widayanti et al. (2014)	MS	n.r.	Shoot tip		Shoot multiplication			*Vanda tricolor*
Tanaka et al. 1975)	VW/Hy	4.7	PLB	5.37 NAA + 44.40 BA	Planlet	800 mg L^{-1} PVP + 0.2% AC		*Vanda*
Khaw et al. 1978)	VW/SH		PLB	1.34-2.69 NAA + 0.44 BA	Apical/axillary bud			*Vanda*
Chaturvedi and Sharma, 1986)	VW		Root/leaf tip	0.57 IAA	PLB/planlet			*Vanda*
Valmayor et al. 1986)	Kn->VW		Flower stalk/bud	4.44 BA + 4.65 Kin	PLB/planlet			*Vanda coerulea*

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The effect of perennial sow-thistle (*Sonchus arvensis*) leaf extract on blood glucose and plasma insulin levels of diabetic mice

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Abstract. *Haryta FS, Listyawati S, Pangastuti A. 2021. The effect of perennial sow-thistle (*Sonchus arvensis*) leaf extract on blood glucose and plasma insulin levels of diabetic mice. Cell Biol Dev 5: 63-69.* The prevalence of diabetes mellitus (DM) has recently increased, thus encouraging drug exploration, especially from natural compounds. One of the potential medicinal plants to reduce blood glucose levels in people with diabetes mellitus is perennial sow-thistle (*Sonchus arvensis*). This study was conducted to determine the effect of perennial *S. arvensis* leaf extract on mice's blood glucose levels and plasma insulin levels induced by streptozotocin. This study used six treatment groups, namely: normal, positive control (acarbose 0.13 mg/20ggBW), negative control (aquadest), and treatment group of perennial *S. arvensis* leaf extract at doses of 50 mg/kgBW, 100 mg/kgBW, and 150 mg/kgBW. The treatments were done by oral administration daily for 14 days. Measurements of blood glucose levels and postprandial plasma insulin levels were measured at 0, 60, and 120 minutes. The test animals were monitored for 14 days, and fasting blood glucose levels were measured on the 7th and 14th days. Data analysis using one-way ANOVA, and if there is a significant difference, it is continued with Duncan's Range Test (DMRT). The results showed that perennial *S. arvensis* leaf extract could reduce postprandial blood glucose elevation and blood glucose levels in diabetic mice, which were monitored for 14 days. The most effective dose is 150 mg/kgBW. Perennial *S. arvensis* leaf extract increased postprandial plasma insulin levels at 60 minutes; the most effective dose is 50 mg/kgBW.

Keywords: blood glucose, diabetes mellitus, perennial sow-thistle leaves, *Sonchus arvensis*, plasma insulin

INTRODUCTION

Diabetes mellitus (DM), also known as a non-communicable disease, is a systemic disease in Indonesia with a high prevalence and mortality rate. Diabetes mellitus is a disorder in which blood glucose levels are consistently higher (hyperglycemia) than normal due to insulin deficiency or inadequate insulin activity (World Health Organization 2003). Insulin therapy, synthetic antidiabetic medicines such as sulfonylureas, biguanides, glinides (Mohammed et al. 2016), metformin, and thiazolidinedione are used to treat diabetes mellitus in general (Meenatchi et al. 2017). However, these synthetic treatments have limitations, including side effects such as hypoglycemia, anemia, weight gain, and heart failure (Acevedo et al. 2017). Considering the impact caused by synthetic drugs, there is research on herbal medicines derived from nature.

The current paradigm in terms of doing the treatment is back to nature, meaning a return to traditional medicine. Plants are a source of phytochemicals for alternative medicine and antidiabetic functional food. One of the medicinal plants that have the potential to treat diabetes mellitus is the perennial sow-thistle (Latin: *Sonchus arvensis*; Indonesian: *tempuyung*). The results of phytochemical screening on perennial *S. arvensis* leaves showed the presence of flavonoids, phenolics, and steroids (Devi et al. 2019). The flavonoid content in perennial *S. arvensis* leaves is dominated by orientin, quercetin, and kaempferol (Khan 2012). The result of the study of percentage inhibition of infusion of perennial *S. arvensis* leaf samples on the activity of α-amylase and α-glucosidase enzymes showed that these plants have potential as antidiabetics, especially in inhibiting the activity of α-amylase enzymes because they contain active flavonoid compounds (Devi et al. 2019). Inhibition of the α-amylase enzyme can delay and prolong carbohydrate digestion, cause a decrease in the rate of glucose absorption and prevent an increase in postprandial blood glucose levels (Sales et al. 2012). An exploratory ethnopharmacology survey in the community in Dawuan District, Subang Regency, West Java Province found that perennial *S. arvensis* leaves were used as a medicinal plant for diabetes or Diabetes mellitus by the local community (Mulyani et al. 2020). This study examined the activity of perennial *S. arvensis* leaves extract as an antidiabetic using a streptozotocin-induced mouse (*Mus musculus*) animal model.

MATERIALS AND METHODS

Place and time

This research was carried out at the Central Laboratory of Pharma-Veterinary (PUSVETMA-Laboratorium Pusat Veteriner Farma) Surabaya from November 2020 to January 2021.

Materials

This study used 24 male (*Mus musculus*) mice with an average weight of 25-30 g, 2-3 months old, healthy, and

from the Central Laboratory of Pharma-Veterinary (PUSVETMA), Surabaya, Indonesia. The code of ethics (Ethical clearance) for the use of test animals has been approved by the Health Research Ethics Committee of the Faculty of Medicine, Sebelas Maret University, with the number: 151/UN27.06.6.1/KEPK/EC/2020 on 21 September 2020. The materials used to manufacture the extract are perennial *S. arvensis* leaf simplicia from the Center for Research and Development of Plants and Traditional Medicines (B2P2TOOT), Tawangmangu, Indonesia, aquadest. The chemicals used were distilled water, acarbose, streptozotocin, citrate buffer pH 4.5, and starch soluble.

Experiment design

This study used a completely randomized design (CRD) with six treatments and 4 replications for each treatment.

Procedures

Test animal preparation

Mice were acclimatized for one week while still being fed and watered, so the mice quickly adapted to the environment and prevented stress.

Making diabetic mice

After 7 days of acclimatization, mice fasted for 8 hours and then measured blood glucose levels and body weight. Next, mice were induced by streptozotocin at a dose of 150 mg/kgBW/day for 2 days intraperitoneally (abdominal cavity) in 5 groups of test animals (except the normal group) (Ria et al. 2015). Streptozotocin (STZ) was dissolved in citrate buffer pH 4.5. On the 3rd day after STZ induction, the blood glucose levels in mice were measured using a glucometer and a strip. Measurement of blood glucose levels by taking blood samples in the veins of the eyes of mice using a microhematocrit tube, the blood that comes out is dripped onto a strip. The blood glucose concentration value will appear in mg/dL units on the screen, and when the blood glucose concentration value is above 200 mg/dL then mice are considered to have diabetes mellitus (Malole and Purnomo 1989). After the mice were declared diabetic, the weight of the mice was also measured and marked on the back and tail of the mice with a permanent marker.

Preparation of 5% perennial Sonchus arvensis *leaf extract with the infundation method*

The S. arvensis leaf simplicia weighed as much as 50 grams; 1,000 mL of distilled water was put into the pot in the infusion, while the outer pot of the infusion was filled with water until it hit or immersed the pot in the infusion. Wait for the distilled water to boil (temperature = 90^{o}C); the perennial *S. arvensis* leaf simplicia is put into a deep pot and boiled for 15 minutes, stirring every 5 minutes. The decoction of the perennial *S. arvensis* leaves is filtered with an anti-bacterial filter while still hot; because the filter results do not reach 1,000 mL, sterile distilled water is added through the filter until it is filled with 1,000 mL. The infusion was stored in a refrigerator at 8 ^{o}C.

Preparation of 5% acarbose suspension and 20% starch solution

Acarbose in tablet form was crushed until smooth, weighed as much as 50 mg, dissolved in 10 mL of sterile distilled water, and poured little by little into the mortar. Next, the acarbose solution was put into a flacon bottle. Finally, the starch solution was made by dissolving 8 grams of starch in 40 mL of sterile distilled water and homogenizing it with a hot plate and a magnetic stirrer until dissolved.

Test animal treatment

The test animals were divided into six treatment groups. The treatment for the test animals was given orally. The grouping of test animals is shown in Figure 1.

Measurement of postprandial blood glucose level

The 0th-minute data was utilized to measure postprandial blood glucose levels, which were obtained by drawing blood from the ocular vein. First, the test animals were given 0.5 mL of soluble starch feed orally, then treated, as shown in Figure 1. Then, using blood from the ocular vein, blood glucose levels were assessed again at 60 and 120 minutes.

Measurement of postprandial plasma insulin levels

Measurement of postprandial plasma insulin levels has the same mechanism and blood sample data as the measurement of postprandial blood glucose levels above. Blood samples were tested using an INSULIN ELISA kit mouse and a microplate reader. The blood samples were centrifuged at 3,500 rpm for 10 minutes, and plasma samples containing insulin (antigen) were obtained above other blood components. Once obtained, the plasma insulin levels were measured according to the manual on the INSULIN ELISA kit mouse.

Monitoring blood glucose levels in test animals for 14 days

The experiment of giving perennial *S. arvensis* leaf extract was carried out for 14 days. Monitoring was carried out by measuring the weight of the mice every day to determine the dose of the test material. After the mice were confirmed to have eaten their food intake (CP 511), they were treated according to the group and transferred to a new cage to prevent any remaining feed. Fasting blood glucose levels were measured on day 7 and day 14, and blood was taken from the caudal vein.

Data analysis

Analysis of the data results using one-way ANOVA (One-Way ANOVA) with a 95% confidence level and continued with the Duncan Multiple Range Test (DMRT) test.

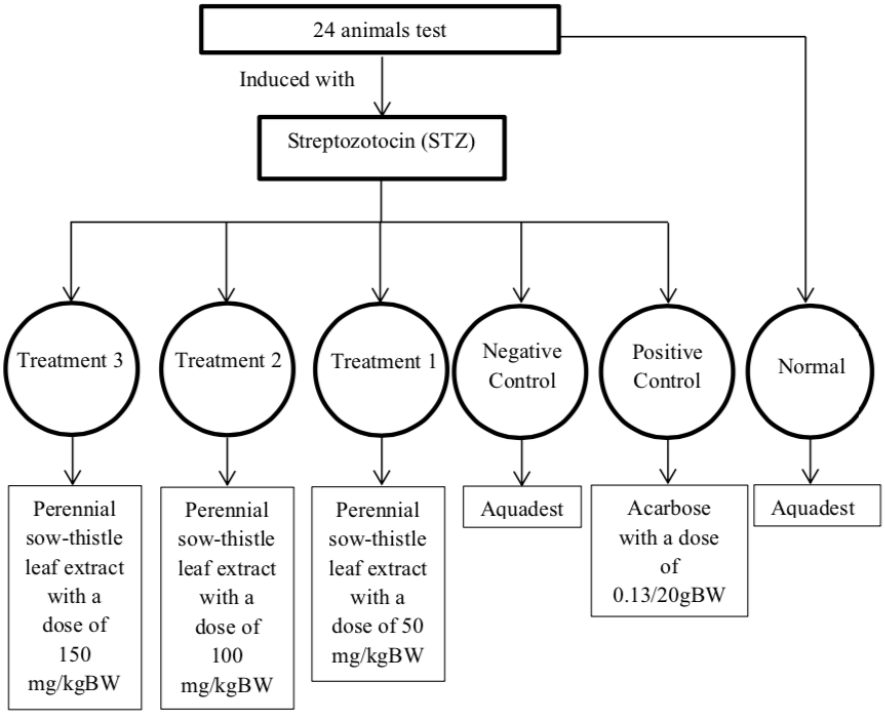

Figure 1. Test animal treatment group

RESULT AND DISCUSSION

Making diabetic mice with streptozotocin

The manufacture of diabetic mice was carried out by inducing streptozotocin at a dose of 150 mg/kgBW/day for 2 days, which can selectively destroy pancreatic β-cells to increase blood glucose levels. Blood glucose levels before and after streptozotocin induction are presented in Table 1.

After the test animals were induced by streptozotocin, blood glucose levels increased in the range of 412 – 539.75 mg/dL. Therefore, the mice were declared to have successfully developed diabetes mellitus (>200 mg/dL) due to the influence of streptozotocin (Malole and Purnomo 1989). The mechanism of action of streptozotocin begins with entry into pancreatic beta cells through the glucose transporter (GLUT-2), which causes DNA damage due to DNA alkylation in the methyl nitrosourea section with the formation of H_2O_2 and inflammatory reactions, resulting in DNA fragmentation. DNA damage activates the enzyme poly (ADP ribose) synthase, an enzyme needed to repair DNA damage. This enzyme requires NAD as its substrate, causing cellular NAD+ and ATP depletion and inhibiting insulin synthesis and secretion. The decrease in ATP synthesis is indicated by dephosphorylation or withdrawal of phosphate groups which produces more substrate for the xanthine oxidase-catalyzed reaction that forms superoxide radicals. The formation of superoxide radicals, resulting in hydrogen peroxide and hydroxyl radicals, is the main cause of pancreatic β-cell damage. In addition, the presence of the N-methyl-N-nitrosourea side chain can release nitric oxide, inhibiting aconitase activity, resulting in mitochondrial dysfunction and causing apoptosis and β-cell necrosis, which eventually causes hyperglycemia (Szkudelski 2001).

The effect of perennial *Sonchus arvensis* leaf extract on postprandial blood glucose levels surges

Based on table 2, there was a spike in blood glucose levels from minute 0 to minute 60. The positive control group had the lowest spike in blood glucose levels because it used acarbose which has a mechanism of action by inhibiting the pancreatic α-amylase enzyme, which works to competitively hydrolyze polysaccharides in the lumen of the small intestine, so there is no spike in blood glucose levels that are too high. The DMRT (Duncan's Multiple Range Test) analysis showed no significant difference between the perennial *S. arvensis* leaf extract group and the positive control group (acarbose). That proves that perennial *S. arvensis* leaf extract has a mechanism of action like acarbose, which can inhibit the α-amylase enzyme in breaking down amylose starch into glucose.

Table 1. Average blood glucose levels of mice before and after streptozotocin induction

Average blood glucose level (mg/dL)	
Before streptozotocin induction	**After streptozotocin induction**
139.50	501.75
120.50	412.00
138.50	539.75
117.50	481.00
138.75	514.50

The α-amylase enzyme has the mechanism of action of initial hydrolysis of the α-(1,4) glycosidic bonds in starch into shorter oligosaccharides with lower molecular weights, such as glucose and maltose. Inhibition of the action of the α-amylase enzyme on the absorption of starch after meals (postprandial) can interfere with or slow down the breakdown of starch, reducing the availability of glucose and maltose and affect the glucose-insulin system, which slows absorption and reduces blood glucose concentrations so spikes can be more controlled. The starch solution is a substrate that can bind to the gap between the carboxyl ends of the A and B domains of the α-amylase enzyme (Souza and Magalhaes 2010). The α-amylase inhibitor can work by imitating the transition position of the pyranosidic unit from the substrate, so it is suspected that the inhibition mechanism is in the form of competitive inhibition (Kim et al., 2008). The flavonoid content in S. arvensis leaves is dominated by orientin, quercetin, and kaempferol (Khan 2012). Tadera et al. (2006) revealed that one of the flavonoid compounds that have the potential to inhibit the α-amylase enzyme is quercetin. The inhibition of the activity of the α-amylase enzyme is related to the hydroxyl group possessed by quercetin. (Yuang et al. 2014). Several studies state that flavonoid compounds also have the inhibitory power of carbohydrate hydrolyzing enzymes. Quercetagetin compounds can inhibit the action of α-amylase enzymes, hydrogen bonds formed between the carboxyl group of the Asp197 side chain on the active site of the α-amylase enzyme and the hydroxyl groups on ring B of quercetagetin can bind covalently, forming a stable bond between quercetagetin and the α-amylase enzyme which results in substrate (starch solution) can no longer bind to the active site of the enzyme so that the product cannot be formed (Piparo et al. 2008). Non-existing products cause glucose absorption in the blood to be controlled so that spikes in blood glucose levels after eating in people with diabetes mellitus can be prevented. The results follow the research by Devi et al. (2019), which states that perennial *S. arvensis* leaves can inhibit the activity of the α-amylase enzyme because it contains phytochemical compounds, particularly flavonoid active compounds.

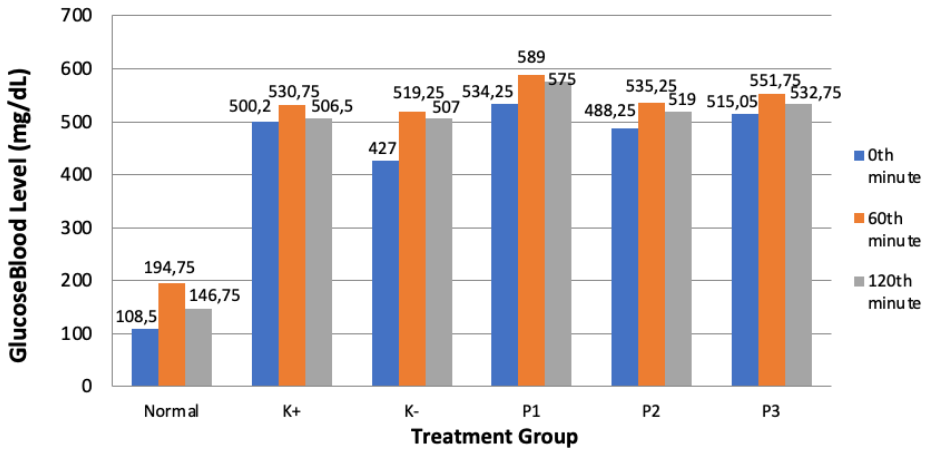

Figure 2 Measurement of postprandial blood glucose levels from 0-120 minutes in mice given starch solution and treatment after the 0th minute. Note: K+ (Positive Control, acarbose), K- (Negative Control, aquadest), P1 (Treatment 1, *S. arvensis* leaf extract with a dose of 50 mg/kgBW), P2 (Treatment 2, *S. arvensis* leaf extract with a dose of 100 mg/kgBW), P3 (Treatment 3, *S. arvensis* leaf extract with a dose of 150 mg/kgBW)

Table 2. Postprandial blood glucose levels at 0-120th minutes

Treatment group	Average difference in blood glucose levels (mg/dl)	
	Minutes 0–60	Minutes 60-120
Normal	86.25[b]	48.00[b]
K + (Acarbose)	30.50[a]	24.25[a]
K – (Aquadest)	92.25[b]	12.25[a]
P1 (*S. arvensis* Leaf extract with a dose of 50 mg/kgBW)	54.75[a]	14.00[a]
P2 (*S. arvensis* Leaf extract with a dose of 100 mg/kgBW)	47.00[a]	16.25[a]
P3 (*S. arvensis* Leaf extract with a dose of 150 mg/kgBW)	36.25[a]	19.00[a]

Note: K + (Positive Control), K – (Negative Control). In the same column, superscripts with different letters indicate significant differences between treatment groups.

The effect of administering perennial *Sonchus arvensis* leaf extract on postprandial plasma insulin levels

Based on the DMRT (Duncan's Multiple Range Test) analysis results, the difference in plasma insulin levels from 0-60 minutes in the normal group significantly differed from the other treatment groups. That is because the normal group is the group with the condition of the test animals in a healthy condition (not having diabetes), so they can raise the highest plasma insulin levels. The positive control and treatment groups of perennial *S. arvensis* leaf extract were not significantly different, but the group differed significantly from the negative control groups. That indicates that the positive control group and the perennial *S. arvensis* leaf extract treatment group had the same ability to increase plasma insulin levels. Still, they were not as effective as the normal group. The negative control group was significantly different from the other treatment groups, indicating that distilled water could not increase plasma insulin levels like other treatment groups. The data analysis results of the difference in plasma insulin levels from 60-120 minutes showed that the negative control significantly differed from the normal treatment group, the positive control group, and the perennial *S. arvensis* leaf extract treatment. That is because the negative control blood glucose level from 60-120 minutes decreased the least compared to the other treatment groups, so plasma insulin in the negative control was still trying to restore blood glucose levels to normal (0th minute).

The administration of streptozotocin is known to cause pancreatic β-cell necrosis, so insulin synthesis and secretion are disrupted. Increased blood glucose levels can stimulate pancreatic cells to secrete insulin. Insulin secretion is biphasic, consisting of phase 1 and phase 2. Phase 1 occurs after stimulation of pancreatic β-cells and lasts briefly (about 10 minutes) and is followed by a continuous phase 2. Phase 2 insulin secretion lasts relatively longer; how high the peak can be determined by how much blood glucose levels are at the end of phase 1 (Jensen et al., 2008). Insulin secretion begins with the entry of glucose through the glucose transporter 2 (GLUT-2) to enter the pancreatic β-cells. Glucose undergoes a process of glycolysis and phosphorylation in the cell, increasing ATP production. The ATP molecules formed to inhibit the ATP-sensitive K+ channel, resulting in the depolarization of the plasma membrane, then the opening of a voltage-gated Ca^{2+} channel. This situation allows the entry of Ca^{2+} ions, causing an increase in intracellular Ca^{2+} ions, which function to activate insulin secretion. Perennial *S. arvensis* leaves can increase insulin secretion at 60 minutes, presumably because the leaves are dominated by flavonoids, especially quercetin and kaempferol, which can increase the induction of insulin secretion by glucose in functioning pancreatic cells (Gupta et al. 2012). Kaempferol can increase ATP production, causing the closure of ATP-sensitive K^+ channels and depolarization of cell membranes (Zhang and Liu 2011). That is supported by research conducted by Bermont et al. (2020), which provides evidence that kaempferol can increase secretion in a pancreatic-cell model by increasing mitochondrial Ca^{2+}. Kittl et al. (2016) reported that there was a 50% inhibition of ATP-sensitive K^+ channels in pancreatic β-cells due to quercetin administration. This effect increases Ca^{2+} levels, which trigger insulin for insulin exocytosis (Bardy et al. 2013).

Correlation of postprandial plasma insulin levels to postprandial blood glucose levels

Based on the results above, the R-value is 0.303, close to 0 and away from 1, which indicates no strong or weak correlation between plasma insulin levels and blood glucose levels. The R^2 value is 9.2%, which indicates that postprandial plasma insulin levels influence 9.2% of postprandial blood glucose levels and other factors influence the remaining 90.8%. Another factor affecting postprandial blood glucose levels in this study is the inhibitory effect of α-amylase enzymes carried out by acarbose (positive control) and flavonoid compounds in the treatment of perennial *S. arvensis* leaf extract to reduce postprandial spikes in blood glucose levels. The regression coefficient value is negative, indicating that the correlation between plasma insulin levels and blood glucose levels goes the opposite. If plasma insulin levels increase, blood glucose levels decrease.

The effect of administering perennial *Sonchus arvensis* leaf extract for 14 days on blood glucose levels

Observation of perennial *S. arvensis* leaf extract for 14 days, counted from the first day of administration of perennial *S. arvensis* leaf extract. This observation was conducted to observe whether perennial *S. arvensis* leaf extract could effectively reduce blood glucose levels for 14 days in diabetic mice. Mice were fed with type CP 511 feed made from corn, soybeans, bran, and wheat. The results of measuring blood glucose levels on the 7th and 14th days are presented in table 4.

The one-way ANOVA test $P<0.05$ showed a significant difference between the treatment groups on the 7th and 14th days. All groups except the negative control group experienced a significant decrease in blood glucose levels because distilled water could not repair pancreatic β-cells that had necrosis due to streptozotocin administration. The positive control group using acarbose on day 14 can reduce blood glucose levels until the mice are no longer in the diabetes category (> 200 mg/dL) (Malole and Purnomo 1989).

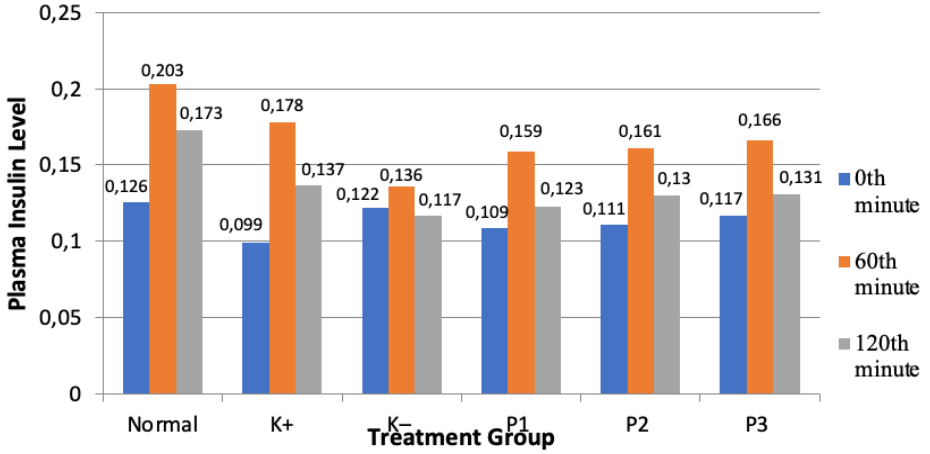

Figure 3. Postprandial plasma insulin levels from 0-120 minutes in mice given starch solution and treatment after 0-minute measurement. Note: K+ (Positive Control, acarbose), K- (Negative Control, aquadest), P1 (Treatment 1, *S. arvensis* leaf extract with a dose of 50 mg/kgBW), P2 (Treatment 2, *S. arvensis* leaf extract with a dose of 100 mg/kgBW), P3 (Treatment 3, *S. arvensis* leaf extract with a dose of 150 mg/kgBW)

Table 3. Postprandial plasma insulin levels at 0-120th minutes

Treatment group	Average difference in blood glucose levels (mg/dl)	
	minutes 0–60	minutes 60-120
Normal	0.076c	0.029b
K + (Acarbose)	0.055b	0.041b
K – (Aquadest)	0.036a	0.018a
P1 (*S. arvensis* Leaf extract with a dose of 50 mg/kgBW)	0.050b	0.036b
P2 (*S. arvensis* Leaf extract with a dose of 100 mg/kgBW)	0.049b	0.031b
P3 (*S. arvensis* Leaf extract with a dose of 150 mg/kgBW)	0.048b	0.035b

Note: K + (Positive Control), K – (Negative Control). In the same column, superscripts with different letters indicate significant differences between treatment groups.

Table 4. Average blood glucose levels on the 7th and 14th days in mice that were treated for 14 days

Treatment group	Average blood glucose level (mg/dL)	
	Day-7	Day-14
Normal	117.00a	105.75a
K + (Acarbose)	299.75b	185.50b
K – (Aquadest)	543.25f	581.25f
P1 (*S. arvensis* Leaf extract with a dose of 50 mg/kgBW)	501.25e	413.75e
P2 (*S. arvensis* Leaf extract with a dose of 100 mg/kgBW)	427.00d	346.00d
P3 (*S. arvensis* Leaf extract with a dose of 150 mg/kgBW)	334.25c	231.75c

Note: K + (Positive Control), K – (Negative Control). In the same column, superscripts with different letters indicate significant differences between treatment groups.

The administration of acarbose in diabetic patients has improved pancreatic-cell function (Tyagita et al. 2021). Increased blood glucose levels increase insulin secretion to improve the function of the remaining pancreatic β-cells (Chen et al. 2014). A significant decrease was also found in the perennial *S. arvensis* leaf extract group at doses of 50 mg/kgBW, 100 mg/kgBW, and 150 mg/kgBW. This decrease was thought to be due to the dominant flavonoid group in the S. arvensis leaf extract. Flavonoids are protective against the damage experienced by pancreatic β-cells and can increase insulin sensitivity. In addition, flavonoids have properties as antioxidants. Antioxidants can reduce Reactive Oxygen Species (ROS); during the formation of ROS, oxygen and electrons will bind to free electrons that come out due to leakage of the electron chain, and the reaction will produce ROS in the mitochondria (Annisa et al. 2014). Free radicals originate from the mechanism of destruction by streptozotocin. Antioxidants in flavonoids can donate hydrogen atoms so that flavonoids are oxidized and bind to free radicals, making free radicals become more stable compounds (Ajie 2015). It was also revealed by Sujono et al. 2014 that as antioxidants, flavonoids work by inhibiting free radicals through redox reactions that can reduce hydrogen donors

and reactive oxygen. One of the compounds from the flavonoid group is quercetin, which can stimulate progenitor cells in the urinary tract pancreas to differentiate to form new islets of Langerhans cells in diabetic rats (Riffai et al. 2012). Research conducted by Khan (2012) states that perennial *S. arvensis* leaves can be used as an effective and safe source of antioxidants as well as ethnomedical that can be developed into drugs because of the presence of flavonoid compounds in the form of kaempferol, myricetin, and quercetin. Based on the results in table 4, the most effective group of perennial *S. arvensis* leaf extract in lowering blood glucose levels is the perennial *S. arvensis* leaf extract at 150 mg/kgBW dose.

This study concludes that perennial *S. arvensis* leaf extract can reduce spikes in postprandial and blood glucose levels for 14 days. The most effective dose of perennial *S. arvensis* leaf extract to reduce blood glucose levels is a dose of 150 mg/kgBW. In addition, perennial *S. arvensis* leaf extract can increase plasma insulin levels at 60 minutes, with an effective dose of 50 mg/kgBW.

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Effect of photoperiod and KNO_3 concentration on the induction and development of potato (*Solanum tuberosum*) microtuber in vitro

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Abstract. *Choirunnisa JP, Wardana R. 2021. Effect of photoperiod and KNO_3 concentration on the induction and development of potato (*Solanum tuberosum*) microtuber in vitro. Cell Biol Dev 5: 70-75.* Potato (*Solanum tuberosum* L.) is a carbohydrate source plant that was developed as an alternative to food diversification. The availability of quality potato seeds is very limited due to the high attack rate of bacteria and viruses. The demand for potato seeds can be fulfilled by developing potato microtuber through the application of plant tissue culture. The purpose of this research was to determine the response of potato microtuber formation with different photoperiods and KNO_3 concentrations. This study design uses a factorial Completely Randomized Design (CRD) with 5 replicates. The first factor is 3 levels of photoperiod (8 hours/day, 12 hours/day, 16 hours/day). The second factor is 3 levels of KNO_3 (1900 mg/L, 2850 mg/L, 3800 mg/L). The study was conducted by observing the age of microtubers initiation, number of shoots, number of roots, number of microtubers, the diameter of microtubers, and wet weight of microtubers were analyzed using SPSS. The results showed that the combination treatment of 8 hours/day photoperiod and KNO_3 concentration of 3800 mg/L could accelerate the initiation of microtubers at 8 DAP (Days After Planting). The concentration of 3800 mg/L KNO_3 can increase the number of roots and microtubers, the diameter of microtubers, and the wet weight of microtubers. The highest diameter of microtubers (17.89 mm) and the highest wet weight of microtubers (278.81 mg) were found in the photoperiod of 16 hours/day. This study concludes that the higher concentration of KNO_3 and the longer photoperiod could be used for the induction and development of potato microtubers, while the short photoperiod could be used to accelerate microtubers initiation

Keywords: KNO_3, microtubers, photoperiod, potato

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the food crops in Indonesia and can be a top priority developed by the public as an alternative to food diversification (Nurjanah 2017). In addition, the development of potatoes as a staple in the snack food industry makes the need for potatoes in Indonesia continues to increase (Aminudin et al. 2014). However, potato production in Indonesia in 2014-2016 decreased from 1247818 tons/ha to 1213041 tons/ha (BPS 2016).

The availability of high-quality potato seeds is very limited; this is one of the factors that caused a decrease in potato production in Indonesia. Potato seeds cannot be fulfilled if only using conventional propagation. Cultivation of potato seeds in conventional propagation needs a long time, namely 100 days, so potato seeds cannot be fulfilled quickly. Tissue culture techniques can produce seeds in a short time with many quantities and are pathogens free (Sagala et al. 2012). Application of tissue culture techniques with potato plants can produce microtubers to support the need for quality seeds. The use of microtubers has several advantages; for example, if the microtubers are transferred to non-aseptic media, they are more resistant and tolerant, and the generated microtubers are uniform and disease-free (Morais et al. 2018).

The factor that can affect the formation of microtubers is photoperiod and nutrient increase in the culture media (Dixit et al. 2011). Culture media nutrients can be increased in-stock solution B of KNO_3 because KNO_3 can increase the size of potato microtubers. KNO_3 in culture media can cause photosynthetic translocation to be more fluent and more quickly into the tubers if the amount of photosynthate produced and absorbed by tubers increases more than the larger potato microtubers (Tessema et al. 2017). The increase in KNO_3 can increase 12% of carbohydrates used to increase potato tubers' weight by 33% (Haddad et al. 2016). In addition, the photoperiod length can increase the intensity of light received by explants to accelerate the process of forming microtubers and affect tuber initiation and the early growth stimulation of microtubers (Sambeka et al. 2012). According to Yasmin et al. (2011), a photoperiod of 16 hours can produce faster induction of microtubers with an average number of microtubers of 1.15.

The purpose of this research was to obtain the optimal concentration of KNO_3 and the right photoperiod for the formation of potato microtubers in a faster time and to produce a size large of microtubers so that they can be used as quality seeds pathogen-free.

MATERIALS AND METHODS

Experimental design

This study design uses a factorial Completely Randomized Design (CRD) with two factors. The first factor is 3 levels of photoperiod, namely 8 hours/day (P1), 12 hours/day (P2), and 16 hours/day (P3). The second factor is 3 levels of KNO_3, namely1900 mg/L (K1), 2850 mg/L (K2), and 3800 mg/L (K3), so it had 9 treatment combinations with 5 replicates. The research was conducted from September 2018 to January 2019 at the Plant Tissue Culture Laboratory, State Polytechnic of Jember, Indonesia.

Field experimental procedure

Making treatment media

The media used are MS media (Murashige and Skoog) which are made according to the Gunawan (1998) stages with a slight modification, for example, the increasing KNO_3 concentration in the stock solution B. The make of MS media can be done by making stock solutions A to H according to the composition of the MS base media, pipetting stock solution A to H according to the standard and put into a glass beaker, then adding the stock solution B (KNO_3) according to treatment and adding the 5 ppm/L BAP, 0.1 ppm/L IAA, 80 g/l sugar and 400 ml aquadest. The mixed-media solution was shaken, the pH was measured to 5.8, then distilled water was added to 1000 ml. The treatment media and 8 g/l jelly powder were put into a pan and cooked on the stove by stirring the solution until it boiled. The media solution was poured into 25 ml culture bottles each, and the bottles were closed with bottle caps. The media was sterilized using an autoclave for 20 minutes at a temperature of 121°C and a pressure of 17.5 psi; then, the media was placed on a culture rack.

Selection of explants

Explants were taken from MS0 media from the tissue culture laboratory and selected with the provisions of a uniform explant age of 12 WAP (Week After Planting) and the same explant stem size. The explants used were two plantlet stem segments with removing the tips and roots.

Subculture

Subculture begins by spraying tools and materials with 70% alcohol, then put into LAFC (Laminar Air Flow Cabinet). The dissecting set was sterilized by heating the bunsen and taking explants from culture bottles using a dissecting set. After the explants were taken, they were placed on a Petri dish, and the explants were cut into single-book micro cuttings with a segment length of 1-2 cm; the tips and roots of the explant were cut using a scalpel. The explants that had been cut were put into the treatment medium in a horizontal position with as many as 2 explants in each bottle; then, the bottles were closed and covered with plastic wrap. The bottles from the subcultures were stored for 3 months on a shelf in the incubation room with a temperature of ± 22°C, RH in a tissue culture room of 55%, and photoperiod time according to treatment. The irradiation on each incubation rack used 2 TL lamps and was set using an automatic timer for 8 hours/day, 12 hours/day, and 16 hours/day.

Observation

Observed variables in this study included the age of microtubers (day-) initiation was observed every day, number of shoots, number of roots, number of microtubers, the diameter of microtubers, and wet weight of microtubers observed at harvest with the age of explants 12 WAP.

Data analysis

Data were analyzed using SPSS (Statistical Product and Service Solution) with Analysis of Variance (ANOVA) and further tested with a Duncans Multiple Range Test (DMRT) level of 5%.

RESULTS AND DISCUSSION

Potato explants at high concentrations of KNO_3 can increase the number of roots, the number of microtubers, the diameter of microtubers, wet weight of microtubers and accelerate the initiation of microtubers. In contrast, explants at low concentrations of KNO_3 can increase the number of shoots. In addition, the long photoperiod can increase the number of shoots, number of roots, number of microtubers, the diameter of microtubers, and weight of microtubers, while the shorter photoperiod can accelerate the initiation of microtubers.

Number of shoots

The parameter of shoot number is a positive indicator in the formation of microtubers; more shoots can potentially form more microtubers. Each explant can grow axillary buds in several nodes or nodes, which can encourage the formation of micro shoots and tubers; according to the statement of Ni'mah et al. (2012), shoots at each node can encourage the formation of microtubers in vitro. The result of the number of shoots analysis is presented in Figure 1. Based on Figure 1, there was no interaction between photoperiod and the addition of KNO_3 to the number of shoots. The longer photoperiod of 16 hours/day could increase the number of shoots by 29% at the concentration of 1,900 mg/L KNO_3, 43% at the concentration of 2,850 mg/L KNO_3, and 30% at the concentration of 3,800 mg/L KNO_3. On the other hand, the highest concentration of 3,800 mg/L KNO_3 can reduce the number of shoots by 33% at various photoperiods.

The longer photoperiod can increase the number of shoots (Figure 1) because the longer photoperiod can increase the photosynthetic process and the results of photosynthesis, namely carbohydrates in explants. Photosynthate results can be used as an energy source for metabolic processes in forming new plant organs such as shoots. Martin et al. (2013) stated that vegetative plant growth, such as shoot growth, was more quickly formed in the 16 hours/day photoperiod due to the increase in photosynthesis, and photosynthesis results in the form of carbohydrates. Aside from that, adding exogenous plant hormones or plant growth regulators of cytokinins such as BAP on culture media can also stimulate shoot growth. BAP is a cytokinin that can affect the number of shoots because it is most active in cell division (Suparaini et al.

2013). Increasing the concentration of KNO_3 can produce a small number of shoots because giving too much K can inhibit N uptake for cell division and plant organ formation. K nutrients too much can be toxic to plants; for example, disrupting the N nutrients absorption has a role in the synthesis of amino acids, cell division, and formation of cells, tissues, and organs of plants (Salli et al. 2016).

Number of roots

The number of roots can be used to indicate plants' ability to absorb nutrients and nutrients. The greatest number of roots can affect the wider range of nutrient absorption so that the roots from the culture medium absorb more nutrients. The nutrients needed by plants can be used as nutrition to increase the plant metabolic processes for plant growth and development (Sarif et al. 2015). The result of the number of roots analyses is presented in Figure 2. Based on Figure 2, there was no interaction between the photoperiod and the addition of KNO_3 to the number of roots. The photoperiod of 16 hours/day could increase the number of roots of potato explants by 27-47% at various concentrations of KNO_3, while the concentration of 3800 mg/L KNO_3 resulted in the highest average number of roots by 19 in the 16 hours/day photoperiod.

The number of roots increased in the longer photoperiod with the higher KNO_3 concentrations (Figure 2) due to the longer photoperiod can increase the synthesis of auxin for root formation. Auxin is a plant hormone that can induce root formation and cell elongation (Lestari 2011). According to Pratiwi et al. (2015), the amount of auxin can increase in long photoperiods for promoting root formation. In addition, the increased concentration of KNO_3 can increase the nutrient content of K and N. The nutrients of K can increase root turgor pressure to absorb nutrients in the media, and the nutrients of N can stimulate the formation of plant vegetative organs so that these two nutrients can support faster root growth. The K nutrients in KNO_3 have a role in stimulating protein formation, increasing root turgor pressure, and stimulating root growth and development (Wahyudi et al. 2015). KNO_3 also contains nitrogen, which is important in plant physiological processes, stimulating root growth and stimulating the growth of plant vegetative organs such as stems and leaves (Leghari et al. 2016). Therefore, the increase of KNO_3 in this research can support root growth and maximize the number of roots.

Age of microtubers initiation

Microtubers are formed at the shoot tip or stolons tip and leaf axils with the characteristics of measuring 1 mm and having yellow, dark green, light green, and white colors. The initiation of microtubers can be influenced by the explant type, the culture medium used, plant hormones, sucrose concentration, and the culture environment (temperature and photoperiod) (Elfiani 2013). Faster formation of potato microtubers in vitro is initiated with swelling of stolons tip growing from the leaf axils due to the growth medium and environmental factors (Nugroho 2013). The result of the age of microtubers initiation analysis is presented in Table 1. Based on Table 1, there was an interaction between photoperiod and the addition of KNO_3 to the age of microtubers initiation. The photoperiod of 8 hours/day with a KNO_3 concentration of 3,800 mg/L resulted in faster initiation of microtubers at 8 DAP, while the photoperiod of 16 hours/day with KNO_3 concentration of 1900 mg/L as a combination treatment with the longest initiation of microtubers at 11 DAP. The age of microtubers initiation at concentration of 3,800 mg/L KNO_3 with 8 hours/day photoperiod was significantly different with 16 hours/day photoperiod.

Table 1. Age of microtubers initiation (DAP) of potato explants cv granola kembang at various photoperiod and KNO_3 concentrations

Photoperiod	KNO_3 concentrations		
	1900 mg/L	2850 mg/L	3800 mg/L
8 hours/day	8.67 ab	8.08 a	8.00 a
12 hours/day	9.78 c	9.08 b	8.75 ab
16 hours/day	10.67 d	10.33 cd	9.83 c

Note: The numbers followed by the different lowercase letters in each column and line showed a significant difference in DMRT level of 5%

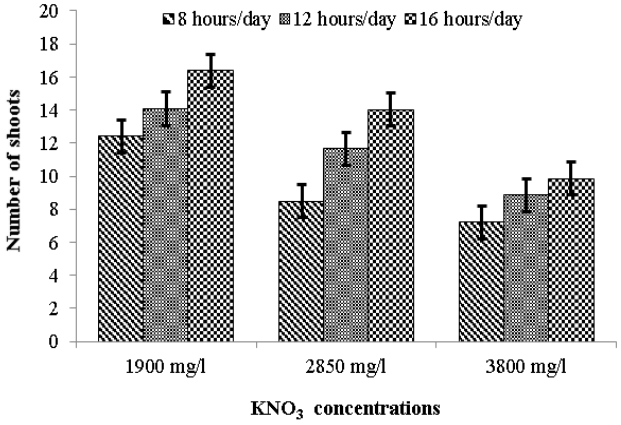

Figure 1. The number of shoots of potato explants cv. granola kembang at various photoperiod and KNO_3 concentrations

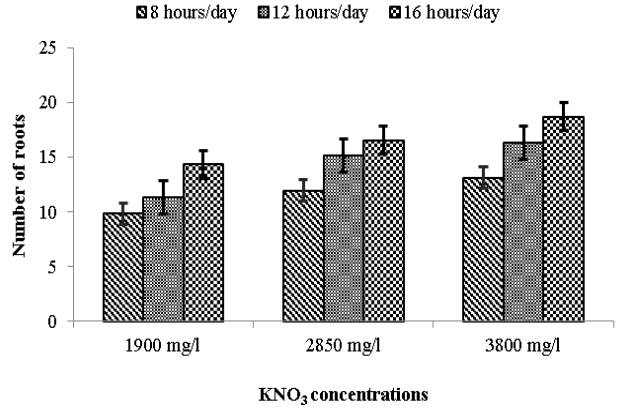

Figure 2. The number of roots of potato explants cv. granola kembang at various photoperiod and KNO_3 concentrations

Microtuber initiation is faster in the shorter photoperiod with higher KNO_3 concentrations (Table 1); this is due to the shorter photoperiod can cause explants to lack light and can produce plant hormones of abscisic acid (ABA) for initiation microtubers. Plants can produce ABA in response to environmental stresses (Verslues 2016), such as the shorter photoperiod for the initiation of microtubers. ABA hormone can inhibit growth and photosynthesis, resulting in carbohydrates being translocated and stored in the energy reserve storage organ, such as tubers. The highest concentration of KNO_3 can accelerate the microtubers' initiation because potassium has a role in accelerating the process of starch synthesis to be stored in storage organs (tubers). According to Utomo and Suprianto (2019), KNO_3 contains 45-46% potassium. Therefore, it has a role in plant physiological processes, such as synthesizing simple sugars, starches, and proteins and accelerating the translocation of carbohydrates to storage organs (tubers). Tubers are storage organs for food or energy reserves such as starch and protein and function as users (sinks) in sucrose metabolism (Turesson et al. 2014).

Number of microtubers

The number of microtubers can be used as an indicator to determine environmental conditions and suitable media for tubers in vitro. Growing environmental conditions and the media used can encourage microtubers initiation and affect the number of micro tubers formed (Hasni et al. 2014). The result of the number of microtubers analyses is presented in Figure 3. Based on Figure 3, there was no interaction between the photoperiod and the addition of KNO_3 to the number of microtubers. The photoperiod of 16 hours/day at the concentration of 3,800 mg/L KNO_3 can increase the number of microtubers by > 30% compared to the photoperiod of 8 hours/day. The concentration of 1,900 mg/L KNO_3 resulted in the maximum number of microtubers by 11-15 micro tubers. The number of microtubers increased by 45% at the KNO_3 concentration of 3,800 mg/L compared to the KNO_3 concentration of 1,900 mg/L.

The number of microtubers increased in the longer photoperiod. At the same time, the higher concentration of KNO_3 can also increase the number of microtubers (Figure 3) because the longer photoperiod can increase the rate of photosynthesis and produce more carbohydrates for the formation of microtubers. The formation of microtubers results from the assimilation process (the rate of photosynthesis) from light as an energy source (Ferreira and Sonnewald 2012). According to (Puangbut et al. 2015), the longer photoperiod can accelerate assimilation and affect the balance and availability of carbohydrates for the initiation and growth of tuber. The increase in KNO_3 concentration can increase the number of microtubers because potassium can increase root growth for nutrient absorption in the media, and adequate plant nutrients can increase the rate of photosynthesis for the formation of microtubers. Potassium can increase the rate of photosynthesis, assimilate yields, and translocate carbohydrates for tuber formation (Fatmawati et al. 2018). One of the products of the photosynthesis of fructans is also needed for tuber formation (Luo et al. 2018).

Diameter of microtubers

The diameter of microtubers can be an indicator of assimilating yields that are translocated to the formation and development of microtubers. The results have been more assimilated and will be translocated to storage organs such as microtubers, which can support the development of microtubers by increasing their size of microtubers. According to Kloosterman et al. (2008), the formation of potato microtubers consists of three stages: induction, initiation, and development of microtubers. The development of potato microtubers also has three stages, namely stalled stolon elongation, subapical swelling in the stolon area, and changes in the diameter of microtubers (Jova et al. 2005). The result of the diameter of the microtubers analysis is presented in Table 2. Based on Table 2, there was an interaction between the photoperiod and the addition of KNO_3 to the diameter of microtubers. The photoperiod of 16 hours/day with a KNO_3 concentration of 3,800 mg/L resulted in the highest microtuber diameter of 17.89 mm, while the photoperiod of 8 hours/day with a KNO_3 concentration of 1,900 mg/L as the lowest diameter of microtuber was 9.23 mm. The concentration of 3,800 mg/L KNO_3 with 16 hours/day photoperiod resulted in significantly different diameters of microtubers with 8 hours/day and 12 hours/day photoperiods. The longer photoperiod and the higher KNO_3 concentration can increase by 48% microtubers diameter.

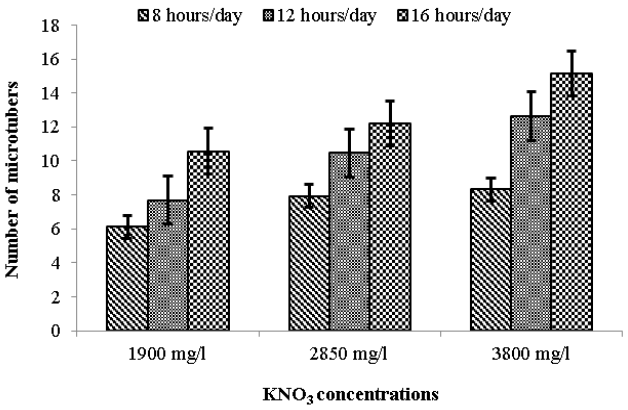

Figure 3. The number of microtubers of potato explants cv. granola kembang at various photoperiod and KNO_3 concentrations

Table 2. The diameter of microtubers of potato explants cv granola kembang at various photoperiod and KNO_3 concentrations

Photoperiod	KNO_3 concentrations		
	1900 mg/L	2850 mg/L	3800 mg/L
8 hours/day	9.23 a	10.42 ab	12.45 c
12 hours/day	11.17 b	12.32 c	14.24 d
16 hours/day	13.10 cd	15.80 e	17.89 f

Note: The numbers followed by the different lowercase letters in each column and line showed a significant difference in DMRT level of 5%

Table 3. The wet weight of microtubers of potato explants cv granola kembang at various photoperiod and KNO_3 concentrations

Photoperiod	KNO_3 concentrations		
	1900 mg/L	2850 mg/L	3800 mg/L
8 hours/day	112.43 a	165.24 ab	199.79 b
12 hours/day	154.36 ab	208.65 b	259.82 cd
16 hours/day	238.45 c	263.88 cd	278.81 d

Note: The numbers followed by the different lowercase letters in each column and line showed a significant difference in DMRT level of 5%

The longer photoperiod and the increased KNO_3 concentration can increase the diameter of microtubers (Table 2); the longer photoperiod can increase the photosynthesis process and accelerate the metabolic process by producing carbohydrates for tuber formation. At the same time, the short photoperiod can inhibit the absorption of water and nutrients in the media for the formation of explant generative organs. According to Golembeski et al. (2014), a short photoperiod on the tissue surface can inhibit the activity of phenolic compounds and cause inhibition of the absorption of water and chemical compounds from the media and the inhibiting of the photosynthesis process. The small size of potato microtubers due to the decreased photosynthesis process and the photosynthate results cannot be distributed optimally in forming generative organs such as tubers (Craze et al. 2018). The increasing KNO_3 concentration can also increase the diameter of microtubers. According to Miao et al. (2016), the process of forming microtubers needs to assimilate the results of carbohydrates. Potassium with sufficient photoperiod can combine CO_2 and water to form sugar that will be converted into ATP (Adenosine Triphosphate) to increase the photosynthesis process (Sulistiani, 2020). In light reactions, ATP is produced from photosynthesis for energy sources in the dark reactions to produce glucose and carbohydrates to form plant organs (Strand et al. 2017).

Wet weight of microtubers

The wet weight of microtubers can be an indicator of assimilating results that have successfully been translocated into tubers. The more assimilates were successfully translocated to the tubers, so the size of the microtubers has bigger. The larger size of microtubers can affect the weight of microtubers due to cell enlargement and cell division continuously so that it can support the development of microtubers (Suh et al. 2014). The result of the wet weight of microtubers analysis is presented in Table 3. Based on Table 3, there was an interaction between the photoperiod and the addition of KNO_3 to the wet weight of microtubers. The photoperiod of 16 hours/day with a KNO_3 concentration of 3,800 mg/L resulted in the highest wet weight of microtubers by 278.81 mg, while the lowest wet weight of microtubers (112.43 mg) was found in the photoperiod of 8 hours/day with KNO_3 concentration of 1900 mg/L. The wet weight of microtubers at 16 hours/day was significantly different with 8 hours/day photoperiod at a KNO_3 concentration of 3,800 mg/L. The increase of KNO_3 concentration from 1900 mg/L to 3,800 mg/L resulted in the wet weight of microtubers being significantly different at each level of the photoperiod.

The wet weight of microtubers increased with the longer photoperiod with the higher KNO_3 concentration (Table 3); this is due to the assimilate results being much more in the long photoperiod with the addition of potassium due to from faster opening and closing of the stomata. The light received by plants for photosynthesis is more when the stomata open and close faster, increasing the photosynthesis process and producing more assimilation. The nutrient K regulates the opening and closing of plant stomata, which can affect the process of receiving light for photosynthesis (Singh et al. 2014). The give of sufficient photoperiod can also suppress the work of auxin to prevent etiolation (cell elongation) in plants (Motallebi et al. 2013). The inhibited cell elongation can result in plants not being etiolated, so assimilated results can be focused on plant growth and development, such as development into microtubers. That is supported by Sarlikioti et al. (2011), that if the production of assimilation is higher, it will be more focused on the development of microtubers. According to Wattimena (1995), the standard as a microtuber propagule has a dry matter percentage of> 14%, a diameter of microtuber by > 5 mm and wet weight of microtubers by > 100 mg/tuber. This research resulted in microtuber wet weight by > 100 mg, so it has fulfilled the microtuber propagule standard.

This study concluded that the initiation of microtubers was faster at the age of 8 DAP with a treatment combination of 8 hours/day photoperiod and KNO_3 concentration of 3,800 mg/L. The photoperiod of 16 hours/day and the concentration of 3,800 mg/L KNO_3 can decrease by 33% shoots number but increase by 47% roots number, 60% microtubers number, 48% microtubers diameter, and 60% microtubers wet weight. The higher concentration of KNO_3 and the longer photoperiod can be used to increase the induction and development of potato microtubers. The short photoperiod can be used to accelerate the initiation of microtubers.

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Effects of mineral levels and leaf extracts of some plants on soil pH and growth rate of selected non-leguminous plants

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Abstract. *Njogu MJ, Murungi JI, Wanjau RN. 2021. Effects of mineral levels and leaf extracts of some plants on soil pH and growth rate of selected non-leguminous plants. Cell Biol Dev 5: 76-89.* The purpose of this study was to determine the effect of growth rate on non-leguminous plants such as wheat (*Triticum aestivum* L.), kale (*Brassica oleracea* var *acephala*), and coriander (*Coriandrum sativum* L.) grown on acidic soils treated with leaves and leaf extracts of plants known to have a high buffering capacity and mineralization capacity, i.e., *Jacaranda mimosifolia* D. Don, *Cordia africana* Lam, *Croton macrostachyus* Hochst. ex Delile, *Vitex keniensis* Turril, *Tithonia diversifolia* (Hemsl.) A.Gray, *Manihot esculenta* Crantz, *Carica papaya* L., and *Helianthus annuus* L. For this investigation, the leaves and leaf extracts were applied to the soil with a pH of 4.75. Leaf extracts and leaves of eight species were combined according to the macronutrient content and mineralization of chosen plants discovered in Nyandarua County, Kenya. Wheat, kale, and coriander growth rates were determined by comparing the dry mass of uprooted seedlings from the trial and control studies every fourteen days for 60 days. Flame photometry, atomic absorption spectrometry (AAS), UV/visible spectroscopy, and turbidimetry were used to determine macronutrients in leaf extracts. The analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) tests were used to analyze the data. For 60 days, the mean pH value of leaf extracts ranged from 4.580.27^{a} to 7.440.04^{d}. K^{+}; 189.95±0.17^{c}, Ca^{2+}; 367.33±0.67^{e}, Mg^{2+}; 114.33^{e}±0.33, $PO4^{3-}$; 55.38±0.23^{f}, NO_3^{-}; 322.25±0.40^{f}, and $SO4^{2-}$; 56.48±0.23^{e} had the greatest mean levels in leaves (µg/g). Macronutrient levels were significantly different in soil treated with leaves and leaf extract (Le), commercial NPK fertilizers (Cf), and untreated soil (Us). Wheat (dry mass) grew at a mean rate of 1.27±0.13^{b} g/wk (Le), 1.26±0.12^{b} g/wk (Cf) and 0.32±0.02^{a} g/wk (Us), while in (height) was 7.29±1.43^{b} cm/wk (Le), 6.20±1.95^{b} cm/wk (Cf), 3.98±0.97^{a} cm/wk (Us). For kales 0.20±0.01^{a} g/wk (Le), 0.30±0.02^{a} g/wk (Cf), and 0.03±0.01^{b} g/k (Us), while in (height) 2.15±0.85^{c} cm/wk (Le), 2.57±0.88^{b} cm/wk (Cf), and 1.04±0.02^{a} cm/wk (Us). The mean growth rate for corianders was 0.16±0.01^{a} g/wk (Le), 0.17±0.02^{a} g/wk (Cf), and 0.10±0.01^{a} g/k (Us), while in (height) 1.85±0.56^{b} cm/wk (Le), 1.86±0.58^{b} cm/wk (Cf) and 0.79±0.18^{a} cm/wk (Us). This study's findings show that the plant's leaves and leaf extracts can be used in place of inorganic fertilizers and should be recommended for agroforestry in low-pH environments.

Keywords: Acidic soils, *Brassica oleracea* var. *acephala*, *Coriandrum sativum*, growth rate, *Triticum aestivum*

INTRODUCTION

Today, man is confronted with the difficulty of providing enough food to feed the world's growing population. Thus, food insecurity has been linked to crop productivity being damaged due to excessive use of commercial inorganic fertilizer on tiny plots of land, which are sometimes the only way to sustain high yields of food crops (Supramudho et al. 2012). Excessive usage of inorganic fertilizer has resulted in a low soil pH, reducing crucial nutrient availability to plants (Ge et al. 2018). As a result, organic stuff decomposes slowly. Additionally, it results in the loss of micronutrients, producing nutritionally deficient food (Handayani et al. 2021). Without addressing the issue, the soil will eventually become unproductive. Organic matter is required to boost the soil's buffering capacity and provide plant nutrients.

Liming is used to raise the pH of the soil; however, it is an expensive process that does not contribute to soil fertility. Therefore, there is a need for an alternative way to inorganic fertilizer and liming material application. In addition, the increased human population has increased not just land usage but also low yields, resulting in forest cover encroachment resulting in reduced rainfall (Lepp and Edwards 1998). As a result, an alternative to inorganic fertilizers that also increases forest cover is required.

The inorganic fertilizers that are routinely used and available primarily feed plants with the principal macronutrient. Nitrogen, phosphorus, and potassium percentage compositions are often listed in the order NPK major (macronutrients). Inorganic fertilizers tend to change the soil pH over time due to their acidic nature (Ge et al. 2018). Reduced soil pH has a detrimental influence on the growth of plants and soil organisms, reducing predicted yields. That is because acid deposition causes important elements such as calcium, magnesium, and potassium to be leached (Murungi 1990). Acidic fertilizers may have little effect on soils with significant buffering capacity. Buffering capacity refers to an ecosystem's ability to maintain a consistent pH regardless of the presence of an acid or a base. Buffering is critical for keeping soil pH from rapidly falling.

Soil with a high proportion of limestone and silicate effectively neutralizes hydrogen ions generated by fertilizers that come into contact with water, allowing the pH to remain normal. Soils, particularly those that cover

granite and igneous rocks, are deficient in buffering material. They have a minor acidic flavor and cannot neutralize the acidic effect. The acidic deposition has been demonstrated not to affect places with a high organic composition, and it is assumed that the decaying products of these locations act as a buffer against the effects of acid (Murungi 1990).

Organic farm manure produced on-site is used for a variety of fertilizing purposes. They are significant in part due to their organic composition. All soils require a source of organic matter for various reasons, including as a carrier of usable energy and nutrients for soil organisms (Liu et al. 2013; Qin et al. 2015; Lestari et al. 2017). Organic manure is primarily composed of rotting plants and is utilized on farms. The quality of manure is determined by the plants utilized to produce it. As a result, it is necessary to identify plants capable of producing high-quality manure. However, the decomposition rate of leaves from diverse plants and the concentrations of macronutrients in their leaves have been examined (Murungi 1990; Njagi 2008). Alien plants have replaced the majority of indigenous trees in the area. It has been noted that some of these exotic plants produce a litter that decomposes slowly and produces acidic decomposition products (Murungi 1990). It is considered that organic matter contributes significantly to the chemical soil's buffering ability (Nur et al. 2019; Taberima et al. 2020). Leaves with a high buffering capacity contain a high concentration of important components (Murungi 1990).

Organic farming is based on cultivating biological diversity in the field to disrupt organisms' habitat and on purposeful soil fertility management and replenishment (Dong et al. 2016; Seufert et al. 2017). The growers use no synthetic pesticides or inorganic fertilizers. On the other hand, organic food is more expensive but healthier to consume because it has significantly fewer synthetic residues than regular food (Kriwy and Mecking 2012). Organic farmers use cover crops, compost, and biologically based soil amendments to feed the soil biota and create organic matter (Serrano et al. 2017; Njoroge et al. 2018), producing robust plants that are resistant to disease and insect predation (Zhang et al. 2019).

There is a need to explore the influence of chosen plants with high macronutrient content and high mineralization on the development rate of selected non-leguminous plants and the pH of acidic soils (Njagi 2008). Therefore, this study analyzed eight plants that showed the possibility of using organic manure as a substitute for inorganic fertilizers.

This study has the following precise objectives: (i) To determine the concentrations of K, N, Mg, P, Ca, and S in selected plants' leaves and leaf extracts, as well as their pH; (ii) To measure the pH of acidic soils before and after treatment with selected plant leaves and leaf extracts; (iii) Every two weeks for a period of 60 days, to monitor the height and dry mass of *Triticum aestivum* L. (wheat), *Brassica oleracea* var *acephala* (kale), and *Coriandrum sativum* L. (coriander) cultivated on soil treated with a combination of leaves and leaf extracts.

MATERIALS AND METHODS

Study area

The study took place in Rurii and Kasuku in Kenya's Nyandarua County. The area is between 1,800 and 2,200 meters above sea level, receives an average annual rainfall of 400-2,200 mL, and has a loam soil type. Nyandarua County is bounded on the north by Laikipia County, on the west by Nakuru County, on the east by Nyeri County, and on the south by Kiambu County. The study location was chosen because of the relatively small parcels of land partitioned into parts for fodder crops, non-leguminous plants for food, and potatoes planted twice a year during the long and short rains. Many farmers use inorganic fertilizers at least twice a season to increase yields (for a base and top dressing). However, some farmers cannot buy inorganic fertilizers and rely on organic fertilizers.

Research design

Randomization was used in the experimental design for leaf gathering. It included using leaves from *Jacaranda mimosifolia* D. Don (E1), *Cordia africana* Lam (L1), *Croton macrostachyus* Hochst. ex Delile (L2), *Vitex keniensis* Turril (L3), *Tithonia diversifolia* (Hemsl.) A.Gray (L4), *Manihot esculenta* Crantz (F1), *Carica papaya* L. (F2), and *Helianthus annuus* L. (F3). The rate of mineralization of these leaves, their effect on the pH of acidic soil, and the effect of combined leaves and leaf extracts on the growth rate of *T. aestivum* (wheat), *B. acephala* (kale), and *C. sativum* (coriander) were also investigated in comparison to the same acidic soil treated with inorganic fertilizer NPK in a control experiment using untreated soil. The research design for this study is shown in Figure 1.

The flow chart in Figure 2 illustrates the process of measuring the growth rate of each non-leguminous plant employed in this research. As in untreated soil, soil treated with commercial NPK fertilizers (Cf), and soil treated with leaves and leaves extracts (Le). Each container contained 30 seeds, from which 5 seedlings were removed every 14 days for 60 days to determine the height (cm) and dry mass (g).

Cleaning of apparatus

All apparatus was washed with a liquid detergent, rinsed, and then immersed for 48 hours in 1:1 analytical grade nitric acid. Next, they were rinsed in aqua regia, tap water, and distilled water many times. After that, the glasswares were dried in a hot oven at 120^{o}C. Prior to each test, the cleaning procedure was repeated.

Reagents and solvents

Throughout the research, analytical grade (AR) reagents from Thomas Baker Chemicals Ltd., Mumbai, India, as well as deionized and distilled water, were employed. In addition, the substances were weighed using a research analytical electronic balance (SHIMADZU, model ATY224, Shimadzu Philippines Manufacturing (SPM) Inc.).

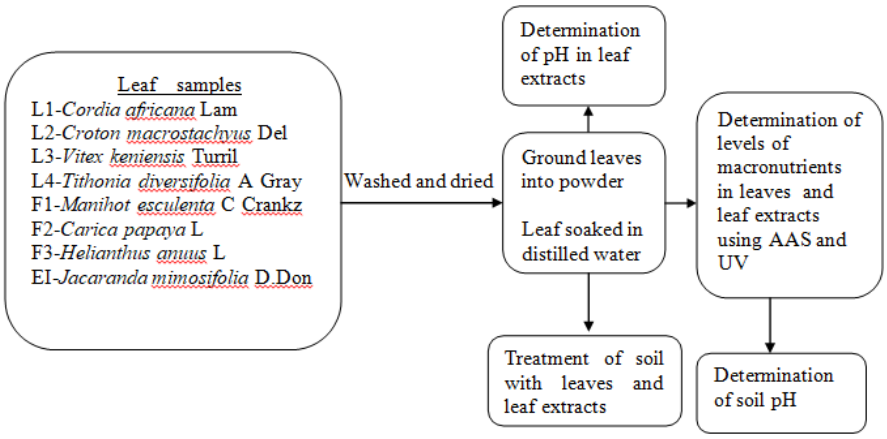

Figure 1. Flow diagram of research design

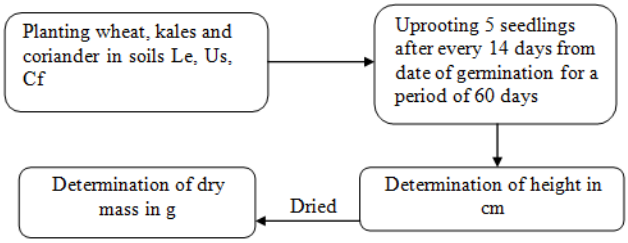

Figure 2. Monitoring of growth rates

Sample collection and sample pre-treatment

Leaves were sampled in April and May 2010 in Nyandarua County. It is rainy, and the plants are vegetative during this season. During this season, trees block sunlight from reaching immature food crops, necessitating their pruning. The leaves and leaf extract acquired for this investigation are used. The sample size was determined using Equation 1 from Gupta and Kapoor (1977):

$$n = \frac{s^2 t_x^2}{d^2 + s t_x^2 / N} \quad \text{.....Eq 1}$$

Where:

n: Sample size

t_x: Critical value of t for (n-1) degrees of freedom and at α: 0:-.05 level of significance, t_x is taken as 1.96.

s: Standard error of the mean

d: Margin of error (the acceptable difference between the sample mean and populationmean), here, taken as 0.04

N: Population size

If *N* is sufficiently large relative to n, Equation 1 reduces to Equation 2

$$n = \frac{s^2 t_x^2}{d^2} \quad \text{..... Eq 2}$$

A systematic sampling design was used to determine the farms from which the plant species would be obtained. First, a random sample technique was utilized to determine six plants from each plant species included in this study. The leaves were then collected from six distinct plants of the same species. Next, the leaves were thoroughly combined from various plants of the same type; sun-dried, placed in clean plastic bags, sealed and labeled suitably. Finally, the leaves plastic bags were placed in nylon sacks and brought to the Kenyatta university's research facility.

In February and March, soil samples were collected in Nyandarua County. After the long dry spell from December to the end of March, the soil is ripe for planting during these months (immediately before the long rains). The farms on which soil samples were gathered were identified through systematic sampling. There are two ways for soil sampling: traverse and zigzag. The traverse approach was used in this investigation. The field's four corners were established, and diagonal sampling was conducted. Twenty soil samples were taken from each farm identified using a soil auger. The soil samples were then properly mixed, and a sub-sample of the mixture was placed in a clean polythene bag for laboratory pH testing. The leftover soil sample was used to monitor the growth rate of the chosen non-leguminous plants. Dry leaves were rinsed with distilled water and sun-dried for three days before being pulverized into a fine powder to determine the macronutrient content. The same powder was used to remediate acidic soils containing non-leguminous plants.

Sample preparation

Preparation of leaf samples for analysis of N, P, K, Ca, and Mg

A bulk of 300 mg was oven dried at 70ºC for four hours. After ground, the leaves were placed in a clean and dry 125 mL "Pyrex" conical flask. A 4 mL quantity of strong sulfuric acid was added, and the flask swirled to soak the sample completely. The flask and contents were heated on an electric hot plate set to "medium" heat for ten minutes. The flask was withdrawn and allowed to cool slightly before adding 10 drops of 30% hydrogen peroxide,

three or four drops simultaneously, to avoid a strong reaction. The flask was spun while the contents remained at the bottom to avoid excessive heating. The flask was allowed to cool before six drops of hydrogen peroxide were carefully added. The flask was then reheated and chilled, and six drops of hydrogen peroxide were added. This procedure was repeated until the solution became colorless. The digest was transferred to a 100 mL volumetric flask and topped off to the desired volume. After that, it was moved to a clean, labeled plastic container and stored until analysis.

Preparation of leaf samples for analysis of sulfur

A quantity of 300 mg grounded leaves was weighed into a dry clean 125 mL "Pyrex" conical flask after being oven dried at 70ºC for 4 hours. A 4 mL quantity of strong nitric acid was added, and the flask swirled to moisten the material completely. The flask and contents were heated on an electric hot plate set to "medium" heat for ten minutes. After cooling the flask, 10 drops of 30% hydrogen peroxide were added, 3-4 drops at a time to avoid a strong reaction. The flask was spun while the contents remained at the bottom to avoid excessive heating. After allowing the flask to cool, six drops of hydrogen peroxide were carefully added and warmed. The chilling process was repeated while adding six drops of hydrogen peroxide until the solution became colorless. The digest was transferred to a 100 mL volumetric flask and diluted to the specified volume. Afterward, the digest solution was transferred to a clean, labeled plastic container and stored in the refrigerator for analysis.

Preparation of stock and standard solutions

Just prior to analysis, stock solutions for various elements were produced from standard solutions. Unless otherwise specified, all reagents used in this investigation were analytical grade. Thomas Baker Chemicals ltd., Mumbai, India, supplied concentrated nitric acid, sulfuric acid, hydrogen peroxide, potassium nitrate, and hydrochloric acid.

The final acid content was kept at roughly 1% throughout the dilutions to maintain the metal in the free ionic condition. Stock solutions were packaged in plastic bottles and clearly labeled. Each time analysis was performed, working standards were produced fresh from stock solutions using serial dilution (Equation 3).

$$C_1V_1 = C_2V_2 \quad \text{.....Eq 3}$$

Where:
C_1: Original concentration
V_1: Original volume
C_2: New concentration
V_2: New volume

A total of six blank samples were digested using the same procedure as the samples to account for background effects from the acids and correct changes resulting from digestion procedures. The elemental concentrations of the elements of interest (K, Ca, and Mg) in each blank sample were determined using an atomic absorption spectrophotometer. According to Christian (2005), their absorbances were measured, and their averages and standard deviations were determined, which were then used to calculate the limit of detection using the formula below:

$$\text{limit of detection} = \frac{3\text{x standard deviation of blank reading}}{\text{Absorbance of standard} - \text{mean absorbance of blanks}} \quad \text{..Eq 4}$$

Magnesium stock and standard solutions

It was necessary to generate a magnesium stock solution (1000 g/g) by dissolving 1.00 g of magnesium ribbon in 300 mL of 1:1 nitric acid and diluting the solution to make it the consistency of a liter with distilled water. A working magnesium standard solution (50 g/L) was prepared by diluting 10 mL of the stock solution to 200 mL and then resolving the solution. The calibration graph was created using a solution containing the following magnesium concentrations: 0, 0.25, 0.5, 1.0, 2.0, 4.0, and 5.0 µg/g of magnesium (in molar concentrations).

Calcium stock and standard solutions

A sum of 2.50 grams of dry calcium carbonate was dissolved in 30 mL of 1 M hydrochloric acid to make the calcium stock solution (1000 µg/g), and the volume was adjusted to 1 L with distilled water at 100ºC for two hours. The calibration curve was generated using standard solutions comprising 0, 5, 15, 20, 30, and 40 µg/g calcium, made by serial dilution of the stock solution.

Potassium stock standard solutions

A potassium stock solution (1000 µg/g) was made by dissolving 1.91 g of dry potassium chloride in approximately 100 mL of distilled water at 100ºC for two hours and diluting to one liter with distilled water. The calibration curve was constructed using potassium standards solutions containing 0, 1, 2, 4, 6, 8, and 10 µg/g potassium.

Nitrogen stock and standard solutions

In order to make a stock solution for nitrogen (with a concentration of 2500 µg/g), 1.179 g of ammonium sulfate was dissolved in water, and the volume was adjusted to one liter with distilled water. The calibration solution consisted of standard nitrogen solutions containing 0, 4, 8, 12, 16, 20, and 24 µg/g nitrogen.

Phosphorous stock and standard solutions

An oven-dried potassium orthophosphate solution (1.10 g) was dissolved in a 250 mL volumetric flask. The volume was then adjusted to the 250 mL mark with distilled water to produce a stock concentration of 1000 µg/g phosphorus. The calibration curve was constructed using standard solutions of phosphorous-containing 0, 1, 2, 3, 4, 5, 6, 7, and 8 µg/g.

Sulfur stock and standard solutions

A stock solution of sulfur (1000 µg/g) was produced by dissolving 2.72 g of potassium sulfate in 500 mL of distilled water and diluting it to 500 mL with distilled water. The calibration curve for sulfur was generated using standard solutions containing 0, 5, 10, 20, 30, 40, and 50 µg/g of sulfur.

Sulfate stock and standard solutions

In order to determine the mineralization of sulfates in a leaf water extract, we prepared a working standard stock solution by dissolving 0.13 g of oven-dried sodium sulfate in distilled water and diluting the solution to one liter with more of the same solution. Sulfates are present in this solution at a concentration of 90 µg/g. The calibration curve was constructed using standard sulfates solutions containing 0, 9, 18, 27, 36, 45, and 54 µg/g.

Sample analysis

Analytical performance of the various instruments to various elements

It was necessary to evaluate the analytical performance of the various equipment used. First, the calibration curves were created by plotting the absorbance of various standards against one another. Next, the slope of the calibration curves was used to determine the sensitivity of the analysis method used. Finally, the detection limit was calculated using the regression equation, as the lowest mean levels obtained by the instrumental signal were equal to the blank signal plus three times the standard deviation of the blank using the regression equation. Tables 1, 2, and 3 present a summary of the findings of this study.

Analytical performance of AAS (Varian spectra AA10) (2005)

Table 1 lists the analytical wavelengths, detection limits, correlation coefficients, and equations of the calibration curves for the measurement of metals in leaves and leaf extracts.

Evaluation of the linearity of the AAS was carried out using the calibration curves that had been established. First, the correlation coefficients (r) were calculated using the absorbance data and the concentration of standards. Then, we estimated the technique detection limits by multiplying the concentration of the provided signals by four times the standard deviations of each of the six blanks. Finally, in order to establish the calibration curves, a plot of absorbance values against the relevant concentration of standards was performed under optimal experimental circumstances.

Analytical performance of the flame photometry

The analytical performance of the flame photometry is shown in Table 2. The linearity of flame photometry was evaluated using the established calibration curves, which were subjected to regression analysis. The correlation coefficients (r) were calculated based on the absorbance values and the concentration of ideal standards employed in the experiments. The concentration of the given signals was computed as the standard deviations of the six blanks times four times the concentration of the given signals. In order to create the calibration curves, a plot of absorbance data against the relevant concentration of ideal standards was performed under optimal experimental conditions.

Analytical performance of T80+ UV/Visible spectrophotometer

The analytical performance of UV/visible spectrophotometry is shown in Table 3. A regression analysis was performed to determine the linearity of the calibration curves that had been established. The calibration curves were plotted using the absorbances and concentrations of ideal standards as input data. To compute the experimental detection limits, we used the regression equation to take the lowest concentration produced by the instrumental signal equal to the blank signal and multiply it by three times the standard deviation of the blank signal. Table 3 displays the findings of the study. The standard solutions created from stock solutions were used to establish UV/visible calibration curves, which were then utilized to determine the amounts of specified analytes in leaves and leaf extracts using the results of the experiments. Based on the data shown in Table 3, it can be stated that the linearity of the calibration curves produced is satisfactory, so accurate measurement can be ensured.

Calibration of pH meter

The pH meter was calibrated using solutions made from pH 4 and 7 tablets.

Table 1. Analytical performance of AAS for the analytes

Analyte	Wavelength (nm)	Range of standards (µg/g)	Sensitivity	Linearity (r2)	Detection limit (µg/g)	Equations for calibration curve
Ca	422.7	0-10	0.0024	0.996	0.038	y=0.01x+0.003
Mg	285.2	0-10	0.0512	0.999	0.032	y=0.084x+0.038

Note: y: absorbance; x: concentration

Table 2. Analytical performance of flame photometry for analytes

Analyte	Range of Standards (µg/g)	Sensitivity	Linearity (r2)	Detection limit (µg/g)	Equations for calibration curve
K	0-10	0.7718	0.996	0.041	y= 0.081x + 0.032

Note: y: absorbance; x: concentration

Table 3. Analytical performance of UV/visible spectrophotometry

Analyte	Range of standards (µg/g)	Sensitivity	Linearity (r2)	Detection limit (µg/)	Equations for calibration curve
Sulfate	0-10	0.0671	0.996	0.038	y = 0.084x + 0.038
Phosphate	0-10	0.5684	0.990	0.003	y = 0.269x + 0.048
Nitrates	0-5	0.2406	0.990	0.048	y = 0.269x + 0.048

Note: y: absorbance; x: concentration

Determination of magnesium by AAS (Varian spectra AA10)

After pipetting a 5 mL aliquot of the wet digested material into a 50 mL volumetric flask, the flask was filled with distilled water. The 285 nm laser beam length was used to nebulize the standards and samples and blank into an atomic absorption spectrophotometer flame to measure absorption. A calibration curve was drawn, and the concentrations of the sample and the blanks were measured (Okalebo et al. 2002).

Determination of calcium by AAS (Varian spectra AA10)

Pipetting a volume of 10 mL of wet digested sample solution into a 50 mL volumetric flask and then filling the flask with distilled water was conducted. A 422 nm laser beam was used to nebulize the standards, samples, and blanks into the flame of an atomic absorption spectrophotometer to measure their absorbance. A calibration curve was drawn, and the concentrations of the sample and the blanks were measured (Okalebo et al. 2002)

Determination of potassium by flame photometry Varian spectra (AA10)

A 5 mL aliquot of the wet digested sample was pipetted into a 50 mL volumetric flask, which was then filled to the top with distilled water. The results are recorded in the table below. A total of 10 mL of the diluted sample and blank were diluted further in a 1:2 ratio, resulting in a final volume of 10 mL. Nebulized into the flame photometer in random order were standards, samples, and a blank sample (Okalebo et al. 2002).

Determination of nitrogen by T80+ UV/Vis spectrometer

Nitrogen determination reagents N1 and N2 were produced. N1 was created by dissolving 34.00 g sodium salicylate, 25.00 g sodium citrate, 25.00 g sodium tartrate, and 0.12 g sodium nitroprusside in one liter of water. In order to make N2, 30.00 g sodium hydroxide was dissolved in water and adjusted to 1 L. Next, sodium hypochlorite was added to a 5 mL aliquot, shaken, and arranged into one liter. The two reagents were employed to treat the samples. First, 5 mL of wet digested samples and blanks were diluted in distilled water at a ratio of 1:9 (v/v). Next, 0.2 mL of diluted wet sample digests, standards, and blanks were pipetted in separate well-labeled test tubes using a micropipette. Each test tube was filled with 5.0 mL of reagent N1 and vortexed, followed by 5 mL of reagent N2. After two hours, the test tubes were allowed to stand, and the absorbance at 650 nm was determined using a PG instruments Ltd T80+ UV/Vis spectrometer (Okalebo et al. 2002).

Determination of phosphorous by T80+ UV/Vis spectrometer

Twelve grams of ammonium molybdate were dissolved in 250 mL warm distilled water (50°C). In a separate experiment, 0.29 g of antimony potassium tartrate was dissolved in 100 mL of distilled water. Both solutions were added to 100 mL of 2.5 M sulfuric acid, well mixed, and diluted with distilled water to two liters. Ammonium molybdate/antimony potassium tartrate was the resultant solution. A 2.11 g of ascorbic acid was dissolved in 400 mL of ammonium molybdate/antimony potassium tartrate solution and thoroughly mixed. A 5 mL sample that had been wet digested was pipetted into a 50 mL volumetric flask. 20 mL distilled water was added, followed by 10 mL ascorbic acid reducing agent and distilled water to make 50 mL. They were then allowed to stand for one hour to allow for complete color development, and their absorbance at 880 nm was determined using a UV/visible spectrophotometer (Okalebo et al. 2002). Additionally, blanks and standards were treated the same way.

Determination of sulfur by UV/Vis spectrophotometry

First, a gelatine-barium chloride solution was prepared by dissolving 0.60 g of gelatin in 200 mL of distilled water and allowing it to stand for 4 to 16 hours in a refrigerator. After bringing the semi-gelatinous fluid to room temperature, 2.00 g of barium chloride was added and well-mixed until dissolved. Next, a 10 mL volume of the digest was pipetted into a 50 mL volumetric flask; 2 mL of gelatin-barium chloride solution was added and diluted with distilled water to the desired concentration. After 30 minutes, absorbance at 420 nm was determined using a UV/visible spectrophotometer (Okalebo et al. 2002). Additionally, blanks and standards were treated the same way.

Determination of mineralization

On the 20th, 40^{th,} and 60th days, 100 mL of deionized water was filtered from the containers where 100 g of leaves had been soaked. It was decided to put the water in a freezer and then analyze it for the presence of potassium, calcium, magnesium, nitrates, phosphorus, and sulfates.

Determination of mineralization of K^+, Ca^{2+}, Mg^{2+}, NO_3^- and PO^{2-}

K^+, Ca^{2+}, and Mg^{2+} concentrations in leaf extracts were determined similarly for wet-digested samples. The concentrations of nitrates and phosphates in leaf extracts were determined in the same manner as for wet-digested

samples, except that the standards for the calibration curve ranged between 0-8 µg/g for nitrates and 0-3.2 µg/g for phosphate.

Determination of mineralization of sulfates by turbidimetry

The following turbidimetric approach was used to determine the quantity of sulfate in leaf water. First, the following solutions were prepared: (i) A glycerol-ethanol combination was prepared by mixing one volume of white glycerol water and two liters of 95% ethanol. (ii) A solution of sodium chloride and concentrated hydrochloric acid was prepared by adding 67.0000 g sodium chloride, and 8 mL concentrated hydrochloric acid to 200 mL water and shaking well.

A 10 mL volume of leaf extract was pipetted into a 50 mL volumetric flask and diluted with distilled water to the desired concentration. After preparation, standards, samples, and blanks (50 mL of each) were placed in clearly labeled beakers. Ten milliliters of glycerol-ethanol and 5 mL of sodium chloride–hydrochloric acid were added. A string bar was inserted, and a beaker was placed on a magnetic stirrer and vigorously swirled. A timer was used to time the addition of 0.02 g of barium chloride. After 60 seconds, the stirrer was stopped, and the absorbance at 420 nm was determined using a UV/visible spectrophotometer. Two samples or standards were run through this procedure once before proceeding to the next, and absorbance was determined within 3-6 minutes of starting the stopwatch (Okalebo et al. 2002).

Calculation of concentrations of elements in the samples

Equation 4 calculated the concentration of critical elements in the samples based on AAS results (readout).

$$\text{Actual concentration } (\mu g/g) = \frac{\text{Concentration } (\mu g/ml) \text{x Volume digested (ml)}}{\text{Weight of dried sample taken (g)}} \quad \text{....Eq 5}$$

When dilution was used, the actual weight was calculated by multiplying the readout values by the dilution factor. The means of the replicate measurements were determined from the actual concentration obtained. The Ca and Mg concentrations in the samples were determined by calculating their means ± standard deviations.

When sample readings were outside the optimum working range, known concentration standards were introduced to bring the sample readings inside this range. The absorbancies of the original sample and the standard were determined. The actual weight of the sample was determined using equation 6 by Skoog et al. (1998).

$$Cx = \frac{A1CsVs}{(A2 - A1)Vx} \quad \text{.....Eq 6}$$

Where:
Cx: Concentration of sample
Cs: Concentration of the standard
A1: Absorbance of the sample before addition of standard
A2: Absorbance of sample after addition of standard
Vs: Volume of standard added
Vx: Volume of sample solution

The pH of leaf extracts analysis

The pH of leaf samples soaked in distilled water was duplicated in the laboratory using a multiline P4 electrochemical analyzer equipped with a suitable multiline pH probe. The pH meter was calibrated using solutions prepared from pH 4 and 7 tablets. Each pill was dissolved in 30 mL distilled water in a 100 mL volumetric flask and agitated until dissolved. Following that, additional distilled water was applied to the spot. Next, a 30 mL sample of the leaf extract was placed in a plastic beaker, immersed with a pH probe, and the pH was determined. The probe was washed multiple times with distilled water following each sample measurement. The pH meter was calibrated again after every ten measurements to guarantee reliable data.

The pH of soil analysis

By weighing 40.00 g of soil into a beaker, the pH of soil treated with powdered leaves and leaf extracts was duplicated. It was then added 100 mL of distilled water mixed for 10 minutes, allowed to stand for 30 minutes, then swirled again for 2 minutes before determining the pH. Next, 30 mL of water was placed in a plastic beaker, the pH probe was immersed, and the pH was determined. After each sample measurement, the probe was washed multiple times with distilled water. Finally, the pH meter was calibrated once again to verify the data was reliable. The identical procedure was performed on soil that had not been treated and soil that had been treated with inorganic fertilizer. The procedure was repeated every 10 days for a total of 60 days.

The analysis of the growth rate of non-leguminous plants

The combined leaves and leaf extract mixture was determined using macronutrient concentrations and their effect on soil pH from this study and an earlier study (Murungi 1990; Njagi 2008). First, to maximize plant nutrition, leaves of *J. mimosifolia* (E1), *M. esculenta* (F1), *C. papaya* (F2), *H. annuus* (F3), *C. africana* (L1), and *T. diversifolia* (L4) were blended in the ratio 3:3:2:2:3:2. Along with treating the acidic soil with mixed leaves, 10 mL of *C. macrostachyus* (L2) and *V. keniensis* (L3) extracts were added at 10-day intervals for 60 days due to their high buffering ability. Next, wheat, kale, and coriander were planted in acidic soil that had been treated with mixed leaves at a mass ratio of 1850:150. In another set, the same plants were grown in acidic soil treated with NPK at a ratio of 1870:130 NPK, and in a third set, the plants were planted in acidic soil that had not been treated.

There were nine replicates of each non-leguminous plant labeled Le with a 2.5 kg capacity for acidic soil treated with combined leaves, nine replicates of each non-leguminous plant labeled FS with a 2.5 kg capacity for soil treated with NPK, and nine containers labeled Us with a 2.5 kg capacity for Us, totaling 27 sets of replicates. On the same day, 30 seeds of each plant were planted 1 cm apart in each container. Five seedlings of each plant were plucked from each container every 14 days from the date of germination for 60 days for each set of duplicates (Le, Cf, and Us). Wheat, kale, and coriander growth rates were

determined using an analytical balance for dry mass and the meter rule for height. Each set of seedlings was washed with tap water and dried under identical conditions, and the average dry mass (g) for each set of experiments was determined. For a total of 60 days, the experiment was reproduced every 14 days.

Data analysis

The data were subjected to statistical analysis, which included the mean, one-way ANOVA, standard deviation, and Student-Newman-Keuls (SNK) test, among other things. The methods are beneficial in determining the dependency of the variables as well as statistically significant differences between the stations (Miller and Miller 1988).

RESULTS AND DISCUSSION

Levels of potassium in leaves and leaf extracts

Through the use of flame photometry, the mean potassium levels in leaves and leaf extracts were determined. Table 4 contains the results of the study.

For potassium, *C. papaya* (F2) had the most potent levels (243.50±0.87 µg/g), while *J. mimosifolia* (E1) had the lowest (71.50±2.29 µg/g). Leaf extracts of *H. annuus* (F3) and *V. keniensis* (L3) showed the highest (18.22±0.38 µg/g) and lowest (10.22±2.38 µg/g) rates of mineralization by the 20th day, respectively. During the 40th day, the leaf extracts of *H. annuus* (F3) and *V. keniensis* (L3) yielded the highest and lowest mean potassium levels, respectively, at 23.43±0.15 and 37.02±1.35. *Helianthus annuus* (F3) 28.76±0.36 and *V. keniensis* (L3) 10.22±2.38 were the highest and lowest, respectively, on the 60th day ($p > 0.05$, SNK test). As the soaking duration increased, potassium mineralization increased in all cases (Table 4).

There have been prior studies showing that *M. esculenta* (F1) 181.67±4.17, *C. papaya* (F2) 243.50±0.87 and *C. africana* (L1) 189.95±5 have high levels of potassium in their leaves, which agree with this study (Lawrence 1990 and Njagi 2008). *Cordia africana* (L1), *M. esculenta* (F1), and *C. macrostachyus* (L2) all had elevated potassium levels in their leaves. That suggests that the leaves of plant species with high mean potassium levels can substitute for expensive bagged inorganic fertilizers. *Carica papaya* (F2) leaves contain 6087 µg/kg of potassium, within the suggested range of 10000-24000 µg/kg for potassium-enriched soil (Lawrence 1990). Plant potassium absorption is 100 g/kg per year (Lawrence 1990). Building protein and photosynthesis, as well as the quality of fruits and the decrease in diseases, are all benefits of potassium. Results show that the combination of leaves and leaf extracts can be employed as a soil supplement for potassium since potassium levels were high in the composite mixture of combined leaves and leaf extracts.

Levels of calcium in leaves and leaf extracts

The mean levels of calcium that were determined are given in Table 5. From 49.33±0.17 µg/g in *J. mimosifolia* (E1) to 367.33±0.67 µg/g *C. africana* (E2), the leaf calcium concentrations differed widely (L1). *Manihot esculenta* (F1) 24.22±0.15, *H. annuus* (F3) 17.16±0.21 and *T. diversifolia* (L4) 28.01±0.24 exhibited the highest rate of calcium mineralization by the 20th day of the extraction. *Helianthus annuus* (F3) was at its maximum level on the 40th and 60th days, whereas *C. africana* (L1) was at its lowest. Soaking time tended to improve calcium mineralization in general (Table 5).

The annual calcium uptake by plants is 170 µg/kg, which is lower than the 1700-24000 µg/kg levels recommended for calcium-enriched soil (Groot et al. 1991). The soil calcium content of *C. africana* (L1), *V. keniensis* (L3), and *M. esculenta* (F1) was found to be 9183.25 µg/kg, indicating that these plants are good organic suppliers of calcium. When calcium levels in soils are too low, leaves can be used in place of more expensive sources such as dolomitic lime or gypsum in order to meet the plant's annual absorption requirements. According to (Njagi 2008), *H. annuus* (F3) had the highest concentration of Ca^{2+}, whereas *C. africana* (L1) had the lowest concentration.

Levels of magnesium in leaves and leaf extracts

Magnesium levels in all leaves and leaf extracts have been analyzed using atomic absorption spectroscopy (AAS). For *T. diversifolia* (L4), the average concentration of Mg^{2+} in leaves was 11.50±0.17 µg/g; for *H. annuus* (L4), it was 114.33±0.33 µg/g (F3). On the 20th day, the leaf extract for *C. africana* (L1) 2.23±0.29 and *H. annuus* (F3) 12.34±0.10 were the lowest and highest, respectively. For *C. africana* (L1), Mg^{2+} levels ranged from 2.43±0.10 to 12.78±0.25 for *V. keniensis* (L3) on day 60. (Table 6).

As a soil supplement for Mg^{2+}, the best trend for *H. annuus* (F3) and *V. keniensis* (L3) was identified throughout the study period. *Helianthus annuus* (F3) and *C. papaya* (F2) showed the highest concentrations of Mg^{2+} in this investigation, which is consistent with the findings of Njagi (2008). In soils where Mg^{2+} deficiency is evident, they were proven to be a good supplement Mg^{2+} concentrations in leaf extracts were nearly identical because it is the only metal found in chlorophyll (Table 6).

Levels of nitrate in leaves and leaf extracts

Nutrient levels in leaves and leaf extracts were measured utilizing UV/visible spectrophotometers. Nitrate levels in leaves ranged from 26.40±0.35 µg/g *H. annuus* (F3) to 322.25±0.40 µg/g *T. diversifolia* (L4) on average (Table 7). *T. diversifolia* (L4) had the highest nitrate-nitrogen 322.25±0.40 levels, whereas leaves from *H. annuus* (F3) had the lowest 26.40±0.35 values, according to the data (Table 4). Leaf extract levels ranged from 0.19±0.20 *V. keniensis* (L3) to 1.37±0.39 *M. esculenta* (F1) during the 20th day of the experiment. Nitrate-nitrogen levels ranged from 0.41±0.18 µg/g *H. annuus* (F3) to 1.39±0.21 µg/g *M. esculenta* (F1) on the 40th day of testing (Table 7).

Table 4. Mean levels (µg/g) of K^+ in leaves and leaf extracts

Plant species	Leaves K^+(mean±SE)	Leaf extracts		
		20th-day K^+(mean±SE)	40th-day K^+(mean±SE)	60th-day K^+(mean±SE)
J. mimosifolia (E1)	71.50±2.29^a	17.32±0.36^f	17.96±0.18^c	19.89±0.31^b
M. esculenta (F1)	181.67±4.17^e	11.20±0.38^d	18.28±0.36^c	18.93±0.15^b
C. papaya (F2)	243.50±0.87^f	13.13±0.36^e	16.99±0.35^c	28.76±0.36^f
H. annuus (F3)	76.23±4.29^a	18.22±0.38^e	23.43±0.15^e	25.04±0.18^d
C. africana (L1)	189.95±5.17^e	13.13±0.28^e	21.12±0.38^d	22.79±0.23^c
C. macrostachyus (L2)	170.83±0.60^d	8.95±0.28^c	16.35±3.33^c	19.25±0.11^b
V. keniensis (L3)	143.83±0.33^c	3.80±0.30^a	7.02±1.35^a	10.22±2.38^a
T. diversifolia (L4)	106.50±0.29^b	6.05±0.37^b	11.52±0.35^b	27.29±0.37^e

Note: Mean values with the same letters within the same column are not significantly different ($p > 0.05$, SNK test)

Table 5. Mean levels (µg/g) of Ca^{2+} in leaves and leaf extracts

Plant species	Leaves Ca^{2+}(mean±SE)	Leaf extracts		
		20th day Ca^{2+}(mean±SE)	40th day Ca^{2+}(mean±SE)	60th day Ca^{2+}(mean±E)
J. mimosifolia (E1)	49.33±0.17^a	4.87±0.32^b	5.23±0.27^b	7.57±0.21^b
M. esculenta (F1)	230.83±0.93^d	24.22±0.15^e	29.66±0.30^d	37.53±0.18^d
C. papaya (F2)	185.50±0.58^c	3.66±0.27^b	5.07±0.27^b	8.74±0.27^c
H. annuus (F3)	316.50±0.50^e	17.16±0.21^d	34.06±0.37^f	39.99±0.18^f
C. africana (L1)	367.33±0.67^e	2.34±0.30^a	2.45±0.21^a	3.42±0.30^a
C. macrostachyus (L2)	193.83±0.73^c	13.86±0.18^c	21.76±0.40^c	38.26±0.27^e
V. keniensis (L3)	114.83±0.67^b	14.21±0.32^c	28.26±0.27^d	38.91±0.40^e
T. diversifolia (L4)	138.00±0.58^b	28.01±0.24^f	31.12±0.24^e	38.33±0.24^e

Mean values with the same letters within the same column are not significantly different($p > 0.05$, SNK test)

Table 6. Levels of Mg^{2+} (µg/g) in leaves and leaf extracts (20^{th}, 40^{th}, and 60^{th})

Plant species	Leaves Mg^{2+}(m±SE)	Leaf extracts		
		20^{th} day Mg^{2+}(m±SE)	40^{th} day Mg^{2+}(m±SE)	60^{th} day Mg^{2+}(m±SE)
J. mimosifolia (E1)	12.67±1.33^a	3.18±0.20^b	3.33±0.25^b	3.35±0.11^b
M. esculenta (F1)	30.17±1.17^c	6.04±0.20^d	6.09±0.33^d	6.12±0.37^d
C. papaya (F2)	41.00±0.17^d	3.75±0.29^b	4.21±0.38^c	4.29±0.20^c
H. annuus (F3)	114.33±0.33^e	12.34±0.10^f	12.38±0.33^f	12.58±0.25^f
C. africana (L1)	28.17±0.17^b	2.23±0.29^a	2.33±0.26^a	2.43±0.10^a
C. macrostachyus (L2)	30.33±1.17^c	5.02±0.26^c	12.23±0.20^f	12.22±0.26^f
V. keniensis (L3)	29.67±0.17^c	11.84±0.11^f	12.69±0.31^f	12.78±0.25^f
T. diversifolia (L4)	11.50±0.17^a	9.17±0.26^e	9.67±0.26^e	9.90±2.33^e

Note: Mean values with the same letters within the same column are not significantly different ($p > 0.05$, SNK test)

Nitrates-nitrogen levels in *T. diversifolia* (L4) were determined to be within the required range of soil enriched with nitrogen 2000-10000 µg/kg since it contained 8056.25 µg/kg. This study found that *T. diversifolia* (L4), a good organic nitrogen source, could supply 100 µg/kg of nitrogen per year to plants. According to what was reported, this agrees with Njagi (2008). Additionally, our findings support the use of *T. diversifolia* (L4) as a top dressing because it decomposes quickly and releases minerals into the water supply (Nancy and Mary 1990).

Levels of phosphate-phosphorous in leaves and leaf extracts

UV/visible spectroscopy was used to estimate the mean amount of phosphate-phosphorous in leaves and leaf extracts; the results are given in Table 8.

Helianthus annuus (F3) to *T. diversifolia* (L4) had mean phosphate-phosphorous levels of 10.60±4.31 µg/g in leaves. Leaf extracts from *C. papaya* (F2) and *H. annuus* (F3) yielded the highest phosphate-phosphorous mineralization levels on the 20^{th} day. Plant leaves of *C. papaya* (F2) had the greatest mean levels of phosphate-phosphorous on the 40^{th} day, whereas those of *H. annuus* (F3) had the lowest. During the 60^{th} day, *M. esculenta* (F1) leaf extract levels averaged 2.03±0.11 µg/g, while *H. annuus* (F3) levels averaged 4.35±0.32 µg/g. Increased soaking time increased the content of leaf extract (Table 8).

Tithonia diversifolia (L4) and *M. esculenta* (F1), which have relatively high phosphorus levels and are readily available as food and fodder crops, can be used to collect phosphorus from the leaves. Phosphorous levels were found to match those of Njagi (2008) closely.

Level of sulfate-sulfur in leaves and leaf extracts

The turbidimetric technique was used to determine the mean concentrations of sulfate-sulfur in leaves and leaf

extracts. Table 9 shows the results. From 12.75±0.40 µg/g in *M. esculenta* (F1) to 56.48±0.23 in *J. mimosifolia* (E1), the leaves of these plants contained various levels of sulfur. By the 20th day, leaf extracts of *V. keniensis* (L3) and *J. mimosifolia* (E1) contained between 0.13±0.24 µg/g and 1.37±0.23 µg/g, respectively. *Helianthus annuus* (F3) varied from 0.27± 0.35 µg/g to 1.49± 0.29 µg/g in *J. mimosifolia* (E1) and 0.52± 0.36 µg/g in *H. annuus* (F3) to 1.99± 0.33 µg/g for *C. macrostachyus* (L2) by the 40th day, respectively (Table 9).

Helianthus annuus (F3) leaves provided 778 µg/kg of sulfur, compared to *C. papaya* (F2) leaves which provided 762 µg/kg. High sulfur demand necessitates higher leaf application because this supply is below recommended amounts of sulfur (1500-1600 µg/kg). Feeding plants with the foliar sulfur deficit is possible by applying leaf extracts. As the soaking duration grew, so did the leaf extract concentration, which is the data in Table 9. This study found that the leaves of *H. annuus* (F3) and *C. papaya* (F2) can be used as an alternative organic source of sulfur. According to Murungi (1990) and Njagi (2008), sulfur levels in this study were extremely low for all species.

Recommended levels of the plant nutrients and plant leaves that are mostsuitable

Detailed macronutrient needs for plant growth and leaf quality are summarized in Table 10. It was shown that *M. esculenta* (F1), *C. papaya* (F2), and *H. annuus* (F3) had the largest occurrences in the supply of macronutrients (P, K and Ca), (Ca, Mg and S) and (K, Mg, and S), sequentially. On the other hand, *Cordia africana* (L1) and *T. diversifolia* (L4) exhibit high quantities of potassium, calcium, nitrogen, and phosphorus. Therefore, the ideal composition is made up of *C. papaya* (F2) for potassium, *H. annuus* (F3) for magnesium and sulfur, *C. africana* (L1) for calcium, and *T. diversifolia* (L4) for nitrogen and phosphorus to ensure a sufficient supply of macronutrients, (Table 10).

Table 7. Mean levels of NO_3^- (µg/g) in leaves and leaf extracts

Plant species	Leaves NO_3^-(m±SE)	Leaf extracts		
		20th day NO_3^-(m±SE)	40th day NO_3^-(m±SE)	60th day NO_3^-(m±SE)
J. mimosifolia (E1)	93.06±0.59^{e}	0.31±0.22^{a}	0.42±0.12^{a}	2.12±0.16^{d}
M. esculenta (F1)	68.17±0.21^{d}	1.37±0.39^{b}	1.39±0.21^{b}	1.84±0.12^{c}
C. papaya (F2)	71.58±0.11^{e}	0.40±0.19^{a}	0.69±0.20^{a}	0.89±0.13^{a}
H. annuus (F3)	26.40±0.35^{a}	0.23±0.11^{a}	0.41±0.18^{a}	1.25±0.17^{b}
C. africana (L1)	40.25±0.02^{b}	0.22±0.22^{a}	0.43±0.19^{a}	2.22±0.27^{d}
C. macrostachyus (L2)	30.47±0.08^{b}	0.21±0.16^{a}	0.43±0.33^{a}	1.23±0.17^{b}
V. keniensis (L3)	50.65±0.24^{c}	0.19±0.20^{a}	0.58±0.23^{a}	0.89±0.19^{a}
T. diversifolia (L4)	322.25±0.40^{f}	0.23±0.26^{a}	0.61±0.16^{a}	2.75±0.29^{e}

Note: Mean values with the same letters within the same column are not significantly different ($p > 0.05$, SNK test)

Table 8. Mean levels (µg/g) of PO_4^{3-} in leaves and leaf extracts

Plant species	Leaves PO_4^{3-} (m±SE)	Leaf extracts		
		20th day PO_4^{3-}(m±SE)	40th day PO_4^{3-}(m±SE)	60th day PO_4^{3-}(m±SE)
J. mimosifolia (E1)	38.90±0.31^{d}	1.42±0.33^{a}	1.83±0.26^{a}	2.08±0.30^{a}
M. esculenta (F1)	49.47±0.29^{e}	1.28±0.14^{a}	1.79±0.17^{a}	2.03±0.11^{a}
C. papaya (F2)	32.37±0.31^{c}	3.05±0.20^{d}	3.45±0.15^{c}	3.72±0.15^{c}
H. annuus (F3)	10.60±4.31^{a}	3.14±0.33^{d}	3.59±0.14^{c}	4.35±0.32^{d}
C. africana (L1)	28.02±1.54^{c}	1.92±0.26^{c}	3.28±0.20^{c}	3.83±0.17^{c}
C. macrostachyus (L2)	18.38±0.31^{b}	1.96±0.30^{c}	2.69±0.33^{b}	3.53±0.14^{c}
V. keniensis (L3)	37.97±0.31^{d}	1.97±0.14^{c}	2.89±0.33^{b}	2.98±0.30^{b}
T. diversifolia (L4)	55.38±0.31^{f}	2.35±0.30^{b}	2.89±0.20^{b}	3.56±0.33^{c}

Note: Mean values with the same letters within the same column are not significantly different ($p > 0.05$, SNK test)

Table 9. Mean levels (µg/g) of sulfate-sulfur in leaf extracts

Plant species	Leaves SO_4^{2-}(m±SE)	Leaf extract		
		20th day SO_4^{2-} (m±SE)	40th day SO_4^{2-} (m±SE)	60th day SO_4^{2-}(m±SE)
J. mimosifolia (E1)	56.48±0.23^{e}	1.37±0.23^{a}	1.49±0.29^{d}	1.94±0.38^{d}
M. esculenta (F1)	12.75±0.40^{a}	0.97±0.40^{a}	1.31±0.33^{c}	1.49±0.18^{c}
C. papaya (F2)	30.49±0.21^{d}	0.68±0.11^{a}	1.33±0.30^{c}	1.94±0.36^{d}
H. annuus (F3)	31.14±0.11^{d}	0.17±0.23^{a}	0.27±0.35^{a}	0.52±0.36^{a}
C. africana (L1)	19.14±0.08^{b}	0.74±0.38^{a}	1.15±0.23^{b}	1.64±0.15^{c}
C. macrostachyus (L2)	19.64±0.24^{b}	0.99±0.11^{a}	1.12±0.29^{b}	1.99±0.33^{d}
V. keniensis (L3)	19.74±0.40^{b}	0.13±0.24^{a}	0.35±0.29^{a}	1.29±0.40^{b}
T. diversifolia (L4)	23.52±0.35^{c}	0.74±0.38^{a}	0.97±0.35^{b}	1.48±0.20^{b}

Note: Mean values with the same letters within the same column are not significantly different ($p > 0.05$, SNK test)

Table 10. Macronutrients and the best sources from the plants studied

Macronutrients	Common range levels in soil µg/kg	Recommended range enriched soil µg/kg	Annual uptake µg/kg	Best leaves for macronutrients
N	2000-4000	2000-10000	100	*T. diversifolia* L4
P	100-5000	400-2000	20	*T. diversifolia* L4, *M. esculenta* F1, *J. mimosifolia* E1 and *V. keniensis* L3
K	400-5000	10000-15000	100	*C. papaya* F2, *C. africana* L1, *M. esculenta* F1, and *C. macrostachyus* L2
Ca	400-35000	1700-24000	170	*C. africana* L1, *H. annuus* F3, and *M. esculenta*
Mg	600-15000	9000-15000	15	*H. annuus* F3 and *C. papaya* F2
S	100-10000	1500-1600	15	*H. annuus* F3 and *C. papaya* F2

Source: Groot et al. (1991)

The pH of leaf extracts

Table 11 shows the pH values of leaf extracts from various plant species, as measured by the researchers. There was no statistically significant difference in the pH of the leaf extract on day 0 ($p > 0.05$, SNK test). On the other hand, by day 15, the value had dropped from 6.67±0.04 to 4.58±0.27. Leaves of *C. africana* (L1) 6.21±0.23, *T. diversifolia* (L4) 6.07±0.23, and *H. annuus* (F3) 6.19±0.32 were extracted on day 15. It was observed on both days 30th and 45th. *Helianthus annuus* (F3) had a pH of 6.25±0.16 while *C. africana* (L1) had a pH of 7.44±0.04. Leaf extracts from *C. africana* (L1) have a pH of 6.21±0.23 to 7.44±0.04, which indicates that they are most suited for treating acidic soils. The pH of *J. mimosifolia* (E1), *C. papaya* (F2), and *M. esculenta* (F1) fluctuated from 4.58±0.27 to 5.49±0.23 throughout the 60 days (Table 11).

By the 60th day, the greatest pH obtained was 7.44 ±0.04 for *C. africana* (L1), and the lowest was 4.58 ±0.27 for *J. mimosifolia* (E1). By the 50th day, the pH of leaf extracts for all species had decreased from 6.67 to below 6.00, except for *H. annuus* (F3), *V. keniensis* (L3), and *T. diversifolia* (L4) (Table 11). The trend in this study for leaf extracts is consistent with that observed in (Njagi 2008; Murungi 1990), where pH decreased and then raised or reduced depending on the type of leaves and the duration of soaking in distilled water.

The pH of *C. africana* (L1) increased over 7.00 over 60 days. The pH of *C. africana* (L1), *J. mimosifolia* (E1), *C. papaya* (F2), and *M. esculenta* (F1) did not increase above 5.50 following the first decline. Several species, including *J. mimosifolia* (E1), *C. africana* (L1), *H. annuus* (F3), *V. keniensis* (L3), *T. diversifolia* (L4), and *C. macrostachyus* (L2), had pH levels rise over 5.50 following the first decline (Figure 3).

Changes in soil pH with the type of soil treatment

It was determined that a pH of 4.75 was used as a control for the acidic soil sample prior to treatment. At the beginning and end of the study, the pH levels of the soil were measured using a pH meter for 60 days. Table 12 lists the pH values of the soils.

It was found that after 50 days, there was a range in the pH of the Us from 4.75±0.02 to 4.02±0.02. pH rose from 4.02 to 4.12 by the 60th day of the study. Leaves and leaf extracts (le) increased the soil's pH from 4.75±0.01 on the 10th day to 6.69±0.01 on the 60th day. SNK test results showed a substantial decrease in pH from 4.75±0.01 to 4.43±0.02 by day 10 and an increase to 4.45±0.02 by day 20 following treatment with NPK. pH decreased from 4.42±0.01 by the 30th day to 4.42±0.01 by the 40th day, and finally declined to 3.98±0.01 by the 50th day and then increased to 4.12±0.01 by the 60th day. Table 12 shows that by the 10th day, the pH of all soils had lowered.

The results corroborate what was stated by (Njagi 2008) that *C. africana* (L1), *C. macrostachyus* (L2), *T. diversifolia* (L4), and *V. keniensis* (L3) increased the pH of the soil to 6.0 or greater for liming. Furthermore, the leaves and leaf extracts of F3, *V. keniensis* (L3), *T. diversifolia* (L4), and *C. africana* (L1) were found to be the most effective in raising the pH of acidic soil, as indicated in Tables 12 and Figure 4.

Table 11. The pH of leaf extracts on days 0, 15, 30, 45 and 60

Plant species	Days				
	0	15	30	45	60
J. mimosifolia (E1)	6.67±0.04^{a}	4.58±0.27^{a}	4.60±0.24^{a}	4.97±0.13^{a}	5.38±0.04^{a}
M. esculenta (F1)	6.67±0.03^{a}	5.40±0.13^{b}	4.87±0.28^{a}	4.90±0.06^{a}	5.12±0.03^{a}
C. papaya (F2)	6.67±0.07^{a}	5.34±0.15^{b}	5.50±0.28^{b}	5.49±0.23^{b}	5.26±0.07^{a}
H. annuus (F3)	6.67±0.08^{a}	6.19±0.32^{d}	6.25±0.16^{c}	5.78±0.06^{c}	6.42±0.08^{c}
C. africana (L1)	6.67±0.04^{a}	6.21±0.23^{d}	7.04±0.25^{d}	7.28±0.05^{f}	7.44±0.04^{d}
C. macrostachyus (L2)	6.67±0.18^{a}	5.49±0.15^{b}	5.85±0.47^{b}	6.27±0.08^{d}	6.00±0.18^{b}
V. keniensis (L3)	6.67±0.05^{a}	5.78±0.24^{b}	5.66±0.06^{b}	5.78±0.28^{c}	6.45±0.05^{c}
T. diversifolia (L4)	6.67±0.20^{a}	6.07±0.23^{d}	6.32±0.47^{c}	6.67±0.12^{e}	6.79±0.20^{c}

Note: Mean values with the same letters within the same column are not significantly different ($p > 0.05$, SNK test)

Table 12. the pH of untreated soil, soil treated with NPK, and soil treated with leaves and leaf extracts

Soils	Days						
	0	10	20	30	40	50	60
Us	4.75±0.02^{a}	4.45±0.01^{b}	4.42±0.01^{a}	4.21±0.01^{a}	4.12±0.01^{a}	4.02±0.02^{b}	4.12±0.01^{a}
Le	4.75±0.01^{a}	4.34±0.02^{a}	4.73±0.01^{c}	5.67±0.02^{c}	5.67±0.02^{c}	6.75±0.01^{c}	6.69±0.01^{b}
Cf	4.75±0.01^{a}	4.43±0.02^{b}	4.45±0.01^{b}	4.42±0.02^{b}	4.42±0.01^{b}	3.98±0.01^{a}	4.12±0.01^{a}

Note: Mean values with the same letters within the same column are not significantly different (p > 0.05, SNK test)

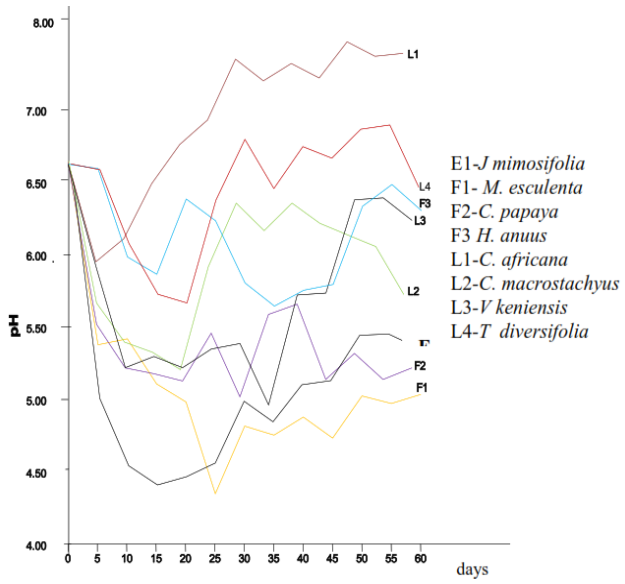

Figure 3. pH trends for leaf extracts

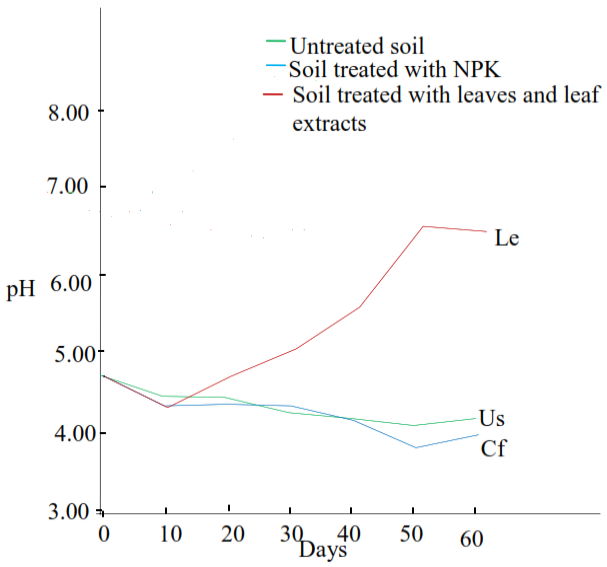

Figure 4. pH trends of soils Le, Cf, and Us

When compared to both NPK-treated and untreated soil, soil treated with leaves and leaf extracts had the highest pH value of 4.75±0.01 after treatment of acidic soil. While NPK treatment had a pH of 3.98 on the 50th day, the soil with mixed leaves and leaf extract had the highest pH. There was a noticeable increase in soil pH after applying leaves and leaf extracts. When NPK was applied, the pH declined to its lowest value of 3.98±0.01 on the 60th day (Table 12 and Figure 4), which suggests NPK was the likely cause of this drop given the Us had a slightly higher pH of 4.02±0.22 by the 50th day (Table 12). The effect of combined leaves and leaf extracts on the growth rate of *T. aestivum* (wheat)

Results of *T. aestivum* (wheat) growth rates in soils treated with leaves and Le, soil treated with Cf, and Us are presented in Table 13. In soil treated with leaves and leaf extracts, *T. aestivum* (wheat) grew at 1.27±0.13 g/wk dry mass and 7.29±1.43 cm/wk height, while in soil treated with Cf, it grew at 1.26±0.12 g/wk dry mass and 6.20±1.9 cm/wk height. The growth rate of *T. aestivum* (wheat) in the Us was 0.32±0.02 g/wk, compared to 1.27±0.13 g/wk in soil treated with leaves and leaf extracts 1.26±0.12 g/wk in soil treated with Cf. Cf leaves and leaf extracts had little effect on *T. aestivum* (wheat) (Table 13). That could occur because the macronutrients in the soil treated with leaves, leaf extracts, and Cf were practically equal, or both contributed appropriate macronutrients.

Those grown in the Us had a mean height of 3.98±0.97 cm/wk, and those grown with Cf had a mean height of 6.20±0.19 cm/wk, respectively (Table 13). The growth rates of *T. aestivum* (wheat) in Us 3.98±0.97 cm/wk, leaves and leaf extracts 7.29±1.43 cm/wk, and Cf 6.20±1.95 cm/wk were significant (p>0.05, SNK test). The macronutrient amounts in the three studies may have contributed to the observed differences in *T. aestivum* height (wheat). The Le included macronutrients that increased *T. aestivum* height (wheat). Figure 5 shows *T. aestivum* (wheat) cultivated on soil with Le, Cf, and Us.

The effect of combined leaves and leaf extract on growth rate *B. acephala* (kale)

For 60 days, researchers tracked the growth of *B. acephala* (kale) in soil treated with Le, Cf, and Us (Table 14). When grown in Cf, *B. acephala* (kale) dry mass increased at a rate of 0.300.02 g/wk, followed by Le, and the least at 0.03±0.01 g/wk when grown in Us. In soil treated with Le, *B. acephala* (kale) grew at a rate of 0.20±0.01 g/week, while Cf at a rate of 0.30±0.02 g/week showed no significant difference (p>0.05 in the SNK test). Growth rates of *B. acephala* (kale) in soil treated with Cf were the fastest, with growth rates of 2.57±0.88 cm/wk, and the slowest, with growth rates of 1.04±0.50 cm/wk in the Us. On the other hand, *Brassica acephala* (kale) grew rapidly in soil treated with Le, with a growth rate of 2.15±0.85 cm/week in soil treated with Le. That is, macronutrient concentrations were high in both the soil treated with Cf and the soil treated with Le, indicating that the soil was supplying enough nutrients to meet plant

demand. According to the findings of this study, *B. acephala* (kale) responded favorably to the leaves and leaf extracts (Table 14), and its heights were comparable to those of plants grown with NPK fertilization. As indicated in Figure 6, *B. acephala* (kale) was grown in soil treated with Le, in Us, and soil treated with nitrogen, phosphorus, and potassium (NPK) fertilizer.

The effect of combined leaves and leaf extract on the growth rate of *C. sativum* (coriander)

To see how *C. sativum* (coriander) grows in soil treated with Le, Cf, and Us, see Table 15. SNK tests showed no statistically significant difference (p>0.05) in the growth rate of the dry mass of *C. sativum* (coriander) growing in soil treated with combined Le 0.06±0.01 g/wk, soil treated with Cf 0.17±0.02 g/wk and Us 0.01±0.001 g/wk. There was a 1.86±0.58 cm/wk growth rate in soil treated with Le and a 0.79±0.57 cm/wk growth rate in the Us (Table 15).

Compared to Us, soil treated with Cf or soil treated with a mixture of Le showed the fastest growth rate for *C. sativum* (coriander) dry mass. For *C. sativum* (coriander), soil treated with Cf grew faster than soil treated with combined Le, because soil treated with combined leaves and leaf extracts took longer to provide macronutrients for the plants. In contrast, Cf delivered them directly to the soil (Figure 2). Therefore, excessive use of Cf on the soil of *C. sativum* plants results in lower yields than using a combination of Cf and leaves and leaf extracts (Figure 7).

Table 13. Mean growth rate of *T. aestivum* (wheat) dry mass (g)

Soil type	Dry mass (Mean±SE) g/wk	Height (Mean±SE) cm/wk
Us	0.32±0.02^{a}	3.98±0.97^{a}
Cf	1.27±0.13^{b}	6.20±1.95^{b}
Le	7.29±1.43^{b}	7.29±1.43^{b}

Note: Mean values with the same letters within the same column are not significantly different (p > 0.05, SNK test)

Table 14. Mean growth rate of *B. acephala* (kale) dry mass (g)

Soil type	Dry mass (Mean±SE) g/wk	Height (Mean±SE) cm/wk
Us	0.03±0.01^{b}	1.04±0.50^{a}
Cf	0.30±0.02^{a}	2.57±0.88^{b}
Le	0.20±0.01^{a}	2.15±0.85^{c}

Note: Mean values with the same letters within the same column are not significantly different (p > 0.05, SNK test)

Table 15. Mean growth rate of *C. sativum* (coriander) in terms of dry mass (g)

Soil type	Dry mass (Mean±SE) g/wk	Height (Mean±SE) cm/wk
Us	0.01±0.01^{a}	0.79±0.57^{a}
Cf	0.17±0.02^{b}	1.86±0.58^{b}
Le	0.06±0.01^{a}	1.85±0.56^{b}

Note: Mean values with the same letters within the same column are not significantly different (p > 0.05, SNK test)

Le **Cf** **Us**

Figure 5. *Triticum aestivum* (wheat) grown in soil treated with Le, Cf, and Us

Le **Cf** **Us**

Figure 6. *Brassica acephala* (kale) grown in soil treated with Le, Cf, and Us

Le **Cf** **Us**

Figure 7. *Coriandrum sativum* (coriander) grown in soil treated with Le, Cf, and Us

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Pre-basic seed production of potato using tissue culture in Katibougou, Mali

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Abstract. *Abdoulaye M, Blay ET, Eleblu JSY. 2021. Pre-basic seed production of potato using tissue culture in Katibougou, Mali. Cell Biol Dev 5: 90-103.* In Mali, the main problem restraining the production and productivity of potatoes is the unavailability of quality seeds at affordable prices and in adequate quantities. This study proposes two experiments on the techniques adopted in Mali's IPR/IFRA plant biotechnology laboratory to meet quantity and quality improvements. That evaluation of the effects of three concentrations of coconut water and two of potassium nitrate on potato plantlets growth in vitro and of three substrates and two physiological ages on potato in vitro plants' establishment, mini tubers production in vivo, and post in vitro growth. The first experiment was a Completely Randomized Design (CRD) with 12 treatments replicated four times. The second was a factorial experiment with two factors (physiological ages: two levels and substrate: three levels) with six treatments replicated four times in a Randomized Complete Block Design (RCBD). The first experiment showed that the culture medium M7 (MS+40 mL/L of coconut water and 250 mg of potassium nitrate) had promoted all plant growth parameters (shoot emergence, number of nodes, leaves, and roots, plant height, and plant fresh and dry weight) after 30 days of in vitro culturing. After 30 days of coconut water (40 mL) and potassium nitrate (250 mg) per liter of MS medium, the lower concentrations had positive effects and significant on all the in vitro growth parameters. The second experiment showed that at 20 days after transplanting in vivo, the post-flask culture substrate S1 (only soil) and the plantlet weaning age of 25 days provided the best plant survival percentage. The substrate S2 (soil and cow dung 2:1) positively affects plant stem length, fresh and dry biomass formation, tuber yield, tuber numbers per plant, stem diameter, and tuber grading size B (diameter of less than 28 mm tubers). The composition S1 substrate (only soil) significantly affected the tubers' stored weight loss (12.50%) within eight weeks. The weaning age was 45 days, and the substrates S2 (soil and cow dung 2:1) and S3 (soil and cow dung 1:1) significantly reduced sprouts' number per eye on tubers and the number of sprouts per tuber. Moreover, the results indicate the coconut water concentration used as a supplement to MS medium should be 40 mL per liter of medium for the better and more rapid growth of potato plantlets in vitro culture. The substrate composition proportion of cow dung should not exceed the soil and cow dung ratio 2:1 for maximum post-transplanting plant re-establishment rate in vivo and rapid maturity of mini-tubers.

Keywords: Potato, *Solanum tuberosum*, tissue culture

INTRODUCTION

The potato (*Solanum tuberosum* L) is an important crop in the sub-Saharan region of Africa, a herbaceous tuberous plant originally from Latin America (MINRESI-IRAD 2012). Potato is the world's main non-cereal food commodity, with global production in 2016 of 19.2 million hectares at around 376.8 million tons, with a yield average of 20 tons/ha. Africa produced 24.5 million tons (FAO 2016). It grew in all Mali regions, with an estimated potato production in 2016 at 210,209 tons in an area of 10,525 hectares with an average yield of 20 tons/ha (FAO 2016).

Mali's market consumes 80% of the volume, and the rest by the sub-region, mainly Ivory Coast, Burkina Faso, Togo, Ghana, and Benin. Ivory Coast, the most important country, absorbed more than 90% of the exported potato (Diakité and Zida 2003). The potato is a cash crop and an important commodity because of its ease of production and high-energy content for millions of farmers in Africa, Latin America, and Asia. It is also important to urban agriculture, which provides employment and food security to 800 million people (FAO 2009). Moreover, it is important to note that in developed countries, particularly in Europe, production has declined in recent years, with a decline of nearly 20%. Still, Africa develops the most, increasing over 55% of production (Vanderhofstadt 2011).

Malian producers generally use two types of seeds: seeds produced by the non-certified farmers themselves and certified seeds imported from Europe; A farmer uses an average of 80% imported and 20% local seed (Diakité and Zida 2003). The timely supply of quality seed (certified seed) in Mali remains dependent on imports from France and Holland (Coulibaly et al. 2002). In Mali, Potato crop production has been increasing since 1973. The cultivated area increased by 4,843 ha from 2010 to 2014. It was estimated at 3,700 ha in 2010 and 8,543 ha in 2014 (Vanderhofstadt 2011). The potato seeds required are 1 to 2.5 tons/ha in Mali, with a minimum of 1,000 kg/ha (1 ton/ha) and an average price of 1,000 CFA francs/kg (BNDA 2014). The potato seeds used by Malian farmers in 2014 were estimated at 8,543 tons, with a turnover for potato seed companies of 8.5 billion CFA francs (around US$14.2 million).

Increased potato production will depend on quality seeds, i.e., varieties resistant to pests and diseases and capable to adapt climate change (FAO 2009). Therefore,

developing potato seeds and cultivation in Mali requires high-yielding varieties and local multiplicate to improve the quality and quantity involving plant biotechnology.

Potato is one of the first significant food crops where the virus has been successfully eliminated using plant biotechnology (Bajaj and Sopory 1986). In addition, tissue culture technology produced disease-free plants and micro tubers, disseminated to the field, and multiplied in many countries (Bajaj and Sopory 1986).

Establishing a seed supply chain requires the production of potato pre-basic seeds in the laboratory. The plant biotechnology laboratory at IPR/IFRA of Katibougou has initiated potato micropropagation since 2000. Some studies prefer to produce potato seed through tissue culture in a 4-year seed production scheme to limit pathogens importation from the in vitro plantlet through generations G0, G1, G2 to G3 seed (Coulibaly et al. 2002).

A study has investigated the coconut water effects on the growth of in vitro Desiree variety potato plantlets in Pakistan (Muhammad et al. 2015). In addition, coconut water has been used as a growth regulator by Overbeek (1941) in culture media for very young *Datura stramonium* embryo development. The cytokinin in coconut water promotes cell division, supplements the chemical components, and promotes plant growth (Jackson et al. 2004).

A study on four physiological ages (20, 30, 40, and 50 days after culturing) in Algeria of potato in vitro plants concluded that the 50-day-old plantlets produced a significantly higher number of mini tubers in vivo than the others (ITCMI 2012). The soil mixes nature used for transplantation could influence the re-establishment of in vitro plantlets in vivo (Anderson 1978). In addition, good aeration of the substrate used for transplanting the in vitro plantlets is important for some species' post-transplanting survival and growth (Gorst et al. 1978).

Unfortunately, the non-availability of quality seeds at affordable prices is the main problem limiting the productivity and production of potatoes in Mali. The seeds imported carry several pathogens, such as bacteria (*Ralstonia solanacearum*) and viruses (PVY and PLRV), that have affected potato production (Vanderhofstadt 2011). In addition, the quality seed supplied does not meet the quantities country's needs.

There is low production through tissue culture of potato pre-basic seed due to a low rate of multiplication of plantlets in vitro. In addition, a general lack of producing pre-basic seed at an identified-appropriate physiological age and a lack of research addressing problems of plantlets in vivo after transplantation associated with post-flask re-establishment under the current climatic conditions in Mali. Therefore, the general objective is to develop reliable protocols for producing potato pre-basic seeds via tissue culture in Mali.

MATERIALS AND METHODS

Study 1

The evaluation of coconut water and potassium nitrate effects on MS medium as supplement components on potato plantlets growth in vitro.

Experimental site

The IPR/IFRA Plant Biotechnology Laboratory, Mali, was the site for this experiment

Plant material

The variety of available potatoes (Sahel) was used as the in vitro plantlets plant material. Therefore, nodal stem cuttings were used and cultured for 30 days in vitro, and one-month-old potato in vitro plantlets was selected as explants (Figure 1).

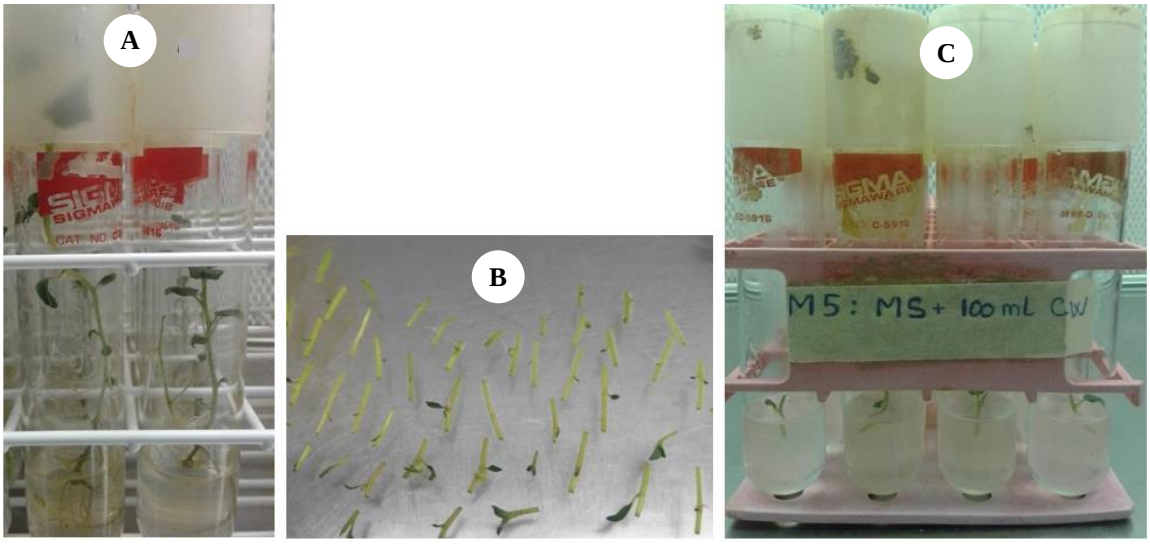

Figure 1. Plant materials. A. Plantlet used as explants; B. Nodal stem cuttings, and C. Explants on media

In vitro growth conditions

In vitro-derived plantlets of the Sahel variety were grown for 30 days at the growth temperature of 24 ± 1°C on 12 culture media and 16 hours of photoperiod. For the micropropagation study, the nodal explants of potato in vitro plants were used. They were obtained by cutting the plants at the internodes. The explant has an axillary bud and leaf, a stem portion 0.5 to 1 cm long, and is placed vertically with the solid culture medium in the culture tube in contact. Each tube contains 10 mL of solidified culture medium.

Culture media

The MS basic medium (Murashige and Skoog 1962) supplemented with 100 mg/L of Myo-inositol, and 30 g/L of sucrose was prepared. Various combinations of coconut water from the mature-dry fruit at two different potassium nitrate (250 mg and 1,000 mg per liter) and three different concentrations (40 mL, 100 mL, and 300 mL per liter) were used in combinations or singly to generate 12 different treatment media. MS medium with no coconut water and Potassium Nitrate (KNO_3) was used as the control.

The pH was adjusted to 5.7 ± 1 of each treatment medium before sterilizing; each treatment medium was solidified with two g/L of Gelrite. Then, the treatment media were sterilized by autoclaving at 115 ± 1°C for 30 minutes.

The twelve (12) media for the experiment were:

MS (control),
M2: MS+250 mg KNO_3;
M3: MS+1000 mg KNO_3;
M4: MS +40 mL/L Coconut Water (CW);
M5: MS+100 mL/L CW;
M6: MS+300 mL/L CW;
M7: MS +40 mL/L CW+250 mg KNO_3;
M8: MS +40 mL/L CW+1000 mg KNO_3;
M9: MS+100 mL/L CW+250 mg KNO_3;
M10: MS+100 mL/L CW+1000 mg KNO_3;
M11: MS+300 mL/L CW+250 mg KNO_3;
M12: MS+300 mL/L CW+1000 mg KNO_3.

In this study, the preparation uses different culture-media. First, the commercial bottles of pre-mixed powders, already available in the laboratory, were used. Then, the "MS Medium" bottle, as prescribed by Murashige and Skoog (1962), contained all the vitamins and mineral salts (macro and micronutrients) (Table 1).

Experimental design

The experiment was a Completely Randomized Design (CRD) with twelve (12) treatments replicated four times.

In vitro experiment layout

A sum of 240 culture tubes was used for the trial, each replication represented by five tubes containing 10 mL of media and one explant.

Data collection

A total of 5 plants were selected per replication per treatment as record plants, and data were collected as follows: (i) Shoot emergence taken ten days after propagating (DAP), (ii) Plant height measured 30 DAP; (iii) Number of leaves per plantlet was taken at 30 DAP; (iv) Number of roots per plantlet taken 30 DAP; (v) Number of nodes per plantlet taken 30 DAP, (vi) Average length of internodes calculated 30 DAP; (vii) Plantlet fresh and dry weight were taken 30 DAP.

Study 2

On evaluation, the weaning age of plantlets and substrate composition affect re-establishment, growth, and mini tuber production of the Sahel variety in vivo.

Experimental site

The experiment was conducted in vivo in a screen house at the IPR / IFRA Plant Biotechnology Laboratory, Mali.

Plant material

The plant material used was plantlets generated in vitro from the Sahel variety. In vitro plantlets at 25 and 45-days characteristics were as follows: (i) 5-7 cm height; (ii) with seven leaves; (iii) and at least four well-developed roots (Figure 2).

In vivo culture substrates preparation and composition

The substrates were composed of three varieties of a mixture of soil and cow dung in (1:0, 2:1, and 1:1). Then, the substrates were steam-sterilized for 30 minutes, cooling, and transferred to the plots and leveled at 7cm depth. The substrate was allowed to settle for 24 hours and well watered before transplanting.

Experimental design

The trial was a factorial experiment with two factors (two levels of physiological ages and three levels of the substrate) with six treatments replicated four times, laid out in a Randomized Complete Block Design (RCBD). A total of 24 plots, each with a plot size of 0.9 m x 0.8 m (0.72 m^2), were used (Table 2).

Table 1. Murashige and Skoog (1962) medium components

Components		**mg/L**
Macronutrients	$CaCl_{22}H_2O$	440
	MgSO42H2O	370
	KH2PO4	170
	NH2NO3	1650
	KNO3	1900
Micronutrients	KI	0.83
	H3BO3	6.20
	MnSO2H2O	22.30
	ZnSO27H2O	8.60
	Na2MgO22H2O	0.25
	CUSO45H2O	0.025
	CoCl26H2O	0.025
Iron	Na2EDTA	37.30
	FeSO47H2O	27.80
Vitamins	Myo-inositol	100
	Nicotinic acid	0.50
	Pyridoxine Hydrochloride	0.50
	Thiamine hydrochloride	0.10
	Glycine	2
Sugar	Sucrose	30000

Table 2. Cropping operations in vivo

Cultivation operations	Period	Dose / Quantity
Plots preparation	before planting	24 plots of 0.72 m^2
First mineral fertilizer application	before planting	300 kg/ha (NPK 17:17:17)
Furadan treatment	before planting	20 kg/ha
Transplantation	20-12-2017	10 cm x 10 cm
Watering	every day	-
Second mineral fertilizer application	15 DAT*	200 Kg/ha (NPK 17:17:17)
Third mineral fertilizer application	25 DAT	200 Kg/ha (NPK 17:17:17)
Fourth mineral fertilizer application	40 DAT	300 Kg/ha (NPK 17:17:17)
1st Earthing up	20 DAT	-
2nd Earthing up	35 DAT	-
3th Earthing up	50 DAT	-
Disease control	26 DAT	Ridomil (fungicide) 5.5 g/plot
Harvest	75 DAT	24 plots
Sorting, sizing, and counting of tubers	At harvest day	-

Note: *DAT: days after transplanting

Treatments

The treatment factors under consideration included the substrate composition on which weaning was carried out and the physiological age of the plantlet at weaning.

Two in vitro plantlet physiological ages at transplanting were evaluated [at 25 days (A1) and 45 days (A2)].

The three substrates used were:

S1 = Soil and Cow dung, 1:0;

S2 = Soil and Cow dung, 2:1;

S3 = Soil and Cow dung, 1:1.

The treatment combinations evaluated in this study are as follows:

T1 = Plantlets at age 25 days+soil and cow dung, 1:0 (A1S1);

T2 = Plantlets at age 25 days+soil and cow dung, 2:1 (A1S2);

T3 = Plantlets at age 25 days+soil and cow dung, 1:1 (A1S3);

T4 = Plantlets at age 45 days+soil and cow dung, 1:0 (A2S1);

T5 = Plantlets at age 45 days+soil and cow dung, 2:2 (A2S2);

T6 = Plantlets at age 45 days+soil and cow dung, 1:1 (A2S3).

The layout of in vivo experiments

This trial needed one screen house to be prepared. Therefore, to accommodate 56 plants were demarcated on each plot size of 0.9 m x 0.8 m. The spacing of 10 cm x 10 cm was adopted, so 1,344 in vitro plantlets were used for the trial (Figure 3).

Agronomic practices in vivo

The in-vitro plantlets were transferred from the culture chamber to the screen house on the same day (from 4 to 6 am) because the outside temperature was lower during this period. First, the in vitro plantlets were directly transferred to growing substrates already disinfected by steam sterilization from the in vitro culture tubes. Then, the plants were transplanted and immediately watered at a spacing of 10 cm × 10 cm.

Storage conditions

Tubers from the ten record plants were stored under natural room conditions, between 24-34°C.

Data collection of in vivo experiments

As record plants, ten plants were selected per treatment; the broader plants were excluded. Data were collected on the following:

Soil and cow dung analysis. Soil and Cow dung samples were seized before the mixture and sent to the laboratory to determine the cow dung's chemical properties (pH and nutrient status) and the physical and chemical properties.

Substrate samples analysis. Samples were seized after harvest from the three substrates used for the in vivo experiments and sent to the laboratory to determine the chemical status of the different spent substrates.

Plant re-establishment rate. The number of the surviving plant was used to calculate the re-establishment rate by counting plants that survived 20 days after transplanting (DAT).

Figure 2. In vitro plantlets at A. 25 days and B. 45 days at weaning age

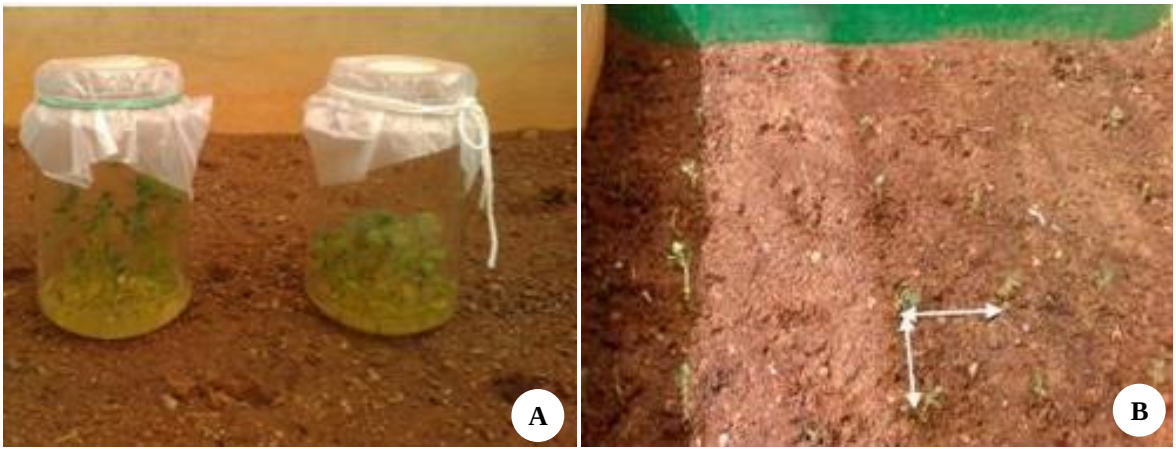

Figure 3. A. In vitro plantlets ready to be transplanted and B. Arrows showing plantlets transplanted at 0.1m x 0.1m spacing

Plant height measurements. Plant height was measured at 15, 40, and 60 DAT. Plant height was measured from the soil level to the top using a tape measure.

Stem diameter measuring. The stem diameter was measured at 15, 40, and 60 DAT; It was taken using a pair of calipers on the plant stem at the collar.

The number of leaves. The number of leaves per plant was taken at 40 DAT by counting the leaves on each record plant (10 plants per treatment) per replication.

Plant fresh weight at harvest. Plant fresh weight was recorded by weighing each of the ten (10) record plants at harvest (75 DAT) per treatment per replication.

Plant dry weight at harvest. The dry weight of the ten (10) record plants after oven drying at 70°C for 48 hours for each treatment was recorded. Before putting them in the oven, the plants were placed in an envelope.

Number of tubers per plant. The number of tubers produced per plant was recorded by calculating the average number of tubers at harvest from the ten (10) record plants per treatment per replication.

Tuber-weight per plant at harvest. Tuber weight per plant was recorded by weighing tubers at harvest for each of the ten record plants per treatment per replication.

Mean tuber weight. The mean tuber weight was taken by dividing the total plants' tuber weight by the number of tubers at harvest for each treatment.

Tuber grading size. The tubers were grouped into three (3) grading size groups from the ten record plants per treatment: (i) Grade A: tubers whose diameter was between 28 and 45 mm; (ii) Grade B: tubers with a diameter of less than 28 mm; (iii) Grade C: tubers with a diameter more than 45 mm. The percentage of each grade of tubers per treatment was recorded after the calculation.

Tuber weight loss in storage. Tubers were stored after harvest for eight weeks; the weight of tubers was taken in storage by weighing tubers from the ten record plants for each treatment every two weeks.

Number of sprouts per tuber in storage. After three months of storage, the number of sprouts per tuber was taken by counting the sprout on the ten stored tubers per treatment.

Sprout number per eye in storage. The number of sprouts per eye by dividing the number of sprouts per tuber by the number of eyes sprouted per tuber for each treatment was taken at three (3) months of storage.

Statistical analysis

Data were analyzed using ANOVA with GenStat (12th Edition). In addition, significant treatment means were separated using Fisher's LSD test at 5% significance.

RESULTS AND DISCUSSION

Effects of culture media on plantlets growth in vitro

Plantlets shoot emergence

The data collected ten days after culturing in vitro on shoot emergence (Table 3) showed significant differences ($p<0.01$) in culture media means. The maximum shoot emergence (100%) was observed in culture media M7 (MS+40 mL/L CW +250 mg KNO3), M8 (MS+40 mL/L CW+1,000 mg KNO3), M10 (MS+100 mL/L CW+1,000 mg KNO3) and M11(MS+300 mL/L CW+250 mg KNO3) after ten days of in vitro culturing. The lowest Shoot emergence (79.17 %), on the contrary, was observed in explants grown on medium M12 (MS+300 mL/L CW+1,000 mg KNO_3).

Plantlets height and internodes length

Data analysis on plantlet height and internode length 30 days after propagating is shown in Table 3, showing significant differences ($P<0.01$) in plant height and internode lengths between the different culture media means. The culture medium M7 (16 cm), with an internode length of 1.4 cm, shows the largest plantlet height. The M8 followed it with 13.5 cm and 1.4 cm internode length, then M4 and M2, respectively (12.8 cm of shoot length and 1.4 cm internode length, and 12.3 cm shoot length and 1.4 cm internode length). Finally, the shortest plant height of 4.8 cm was observed in the M12 with an internode length of 0.9 cm.

Number of leaves

Table 4 presented the data on the number of leaves at 30 days after propagating in vitro; in mean leaves numbers on the various culture media, highly significant differences ($p<0.01$) were observed. However, the M7 plantlets showed the highest mean number of leaves (13.45). M8 followed it with a mean of 11.7 leaves, then M4 of 11 leaves. There was no observed significant difference in mean leaves number between M2: MS+250 mg/L of KNO3, M5: MS+100 mL/L of coconut water, and M3: MS+1000 mg KNO3 with the respective leaf means of 10, 10, and 9.85. Finally, the least number of leaves was produced on M12: MS+300 mL/L CW+1000 mg KNO3 with 7.35 leaves.

Number of roots

Statistical analysis on plant root numbers after 30 days of in vitro propagation is presented in Table 4 and shows highly significant differences ($p<0.01$) between the 12-culture media. In culture media M7 and M8 observed the highest root number per plant, with nine roots each, and M3 recorded the least root number (1.2).

Number of nodes

Analysis of plant nodes number after 30 days of in vitro culture is presented in Table 4 and shows significant differences ($p<0.01$) between the culture media means. The M7 showed the highest mean number of nodes (11.45) per plant. M8 followed it with a mean of 9.65 nodes, next M4: MS+40 mL/L Coconut Water with nine nodes. On the contrary, the on M12 (5.35).

Plant fresh and dry weight

In mean plant fresh and dry weights between the different culture media, the data recorded 30 days after propagating (DAP) in vitro revealed significant differences ($p<0.01$). There were observed the highest plant fresh and dry weights (545.50 and 36.40 mg) on culture medium M7 (MS+40 mL/L of coconut water+250 mg of potassium nitrate), next by M2 (MS+250 mg/Liter of potassium nitrate) with the means of 364.50 mg for the fresh weight and 23.15 mg for the dry weight. While the M12: MS+300 mL/L of coconut water+1,000 mg/Liter of potassium nitrate with 99.20 mg for mean fresh and 13.20 mg for the mean dry weight (Figures 4, 5, 6, and 7) recorded the lowest.

According to Fisher's LSD test at 5% significance, letters represent significant differences among media

Plantlets weaning age and substrate composition effects on mini tubers production

Soil properties

The laboratory analysis shows that the soil has a sandy loam texture and a pH of 7.68, of the physical and chemical properties of the soil used as the basic substrate in the experiment (Table 5).

Cow dung properties

The laboratory analysis shows that the pH is 7.26, with a significant amount of NPK of the chemical properties of the cow dung used in the substrate composition for the experiment (Table 6).

Table 3. Media effect on shoot emergence after 10 days, plant height, and internode length after 30 days of in vitro culturing

Culture media	Shoot emergence %	Plant height (cm)	Internode length (cm)
M1	83.33 ^{bc}	10.00 ^{d}	1.43 ^{b}
M2	91.67 ^{abc}	12.30 ^{c}	1.54 ^{a}
M3	95.83 ^{ab}	9.90 ^{d}	1.27 ^{c}
M4	83.33 ^{bc}	12.78 ^{c}	1.42 ^{b}
M5	95.83 ^{ab}	8.68 ^{e}	1.09 ^{de}
M6	95.83 ^{ab}	6.93 ^{g}	0.99 ^{ef}
M7	100 ^{a}	16.00 ^{a}	1.40 ^{b}
M8	100 ^{a}	13.53 ^{b}	1.40 ^{b}
M9	91.67 ^{abc}	8.95 ^{e}	1.18 ^{cd}
M10	100 ^{a}	7.60 ^{f}	1.18 ^{cd}
M11	100 ^{a}	5.45 ^{h}	0.92 ^{f}
M12	79.17 ^{c}	4.80 ^{i}	0.90 ^{f}
p-value	0.039	<.001	<.001

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. p-value= probability value

Table 4. Comparison of the effect of different media on No. of leaves, roots, and nodes of in vitro-grown potato plantlets after 30 days

Culture media	No. of leaves	No. of roots	No. of nodes
M1	9.00 ^{ef}	5.10 ^{d}	7.00 ^{ef}
M2	10.00 ^{d}	6.35 ^{c}	8.00 ^{d}
M3	9.85 ^{d}	6.95 ^{b}	7.85 ^{d}
M4	11.00 ^{c}	6.80 ^{b}	9.00 ^{c}
M5	10.00 ^{d}	4.00 ^{f}	8.00 ^{d}
M6	9.00 ^{ef}	1.60 ^{h}	7.00 ^{ef}
M7	13.45 ^{a}	9.00 ^{a}	11.45 ^{a}
M8	11.65 ^{b}	9.00 ^{a}	9.65 ^{b}
M9	9.60 ^{de}	4.00 ^{f}	7.60 ^{de}
M10	8.45 ^{fg}	4.50 ^{e}	6.45 ^{fg}
M11	7.95 ^{gh}	2.20 ^{g}	5.95 ^{gh}
M12	7.35 ^{h}	1.20 ^{i}	5.35 ^{h}
p-value	<.001	<.001	<.001

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. p-value= probability value

Table 5. Soil properties before the substrate mixture

Soil properties	Amount
Texture: Sand (%)	72.00
Silt (%)	26.00
Clay (%)	2.00
pH in water	7.68
pH in KCl	6.96
Organic carbon (%)	0.65
Nitrogen total (%)	0.05
Phosphorus assimilable (ppm/100g)	78.32
Potassium assimilable (mg/100g)	10.64

Note: Laboratory SEP-IER, Mali

Table 6. Chemical properties of the cow dung used

Cow dung properties	Amount
pH in water	7.26
pH in KCl	6.63
Nitrogen (N %)	0.81
Phosphorus (P2O5 %)	0.89
Potassium (K2O %)	0.25

Note: Laboratory SEP-IER, Mali

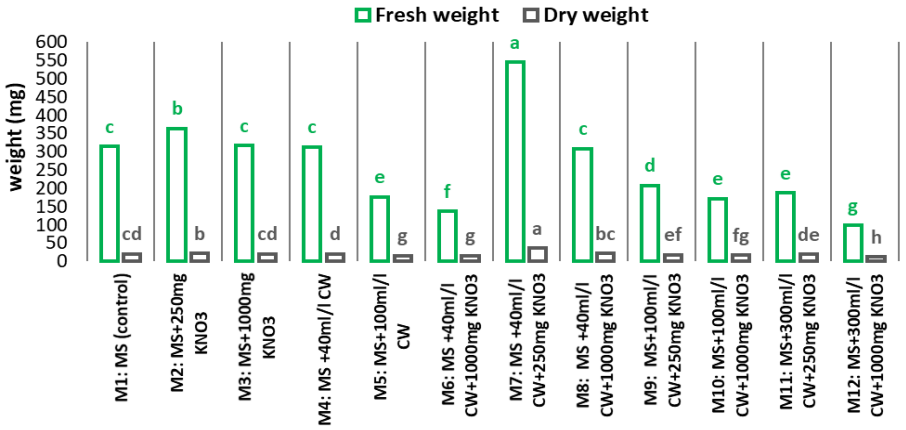

Figure 4. Effect of media on plantlet fresh and dry weights 30 days after culturing in vitro

Figure 5. Development of in vitro plantlets 30 days after culture on media M1, M2, M3, and M4

Figure 6. Development of in vitro plantlets 30 days after culture on media M5, M6, M7, and M8

Figure 7. Development of in vitro plantlets 30 days after culture on media M9, M10, M11 and M12

Chemical properties of spent substrates

The laboratory analysis of the three substrates of the physical and chemical properties used in the experiment shows that the pH of the three substrates tested was reduced compared to the initial state of the soil before a mixture of substrates and planting (Table 7).

Plant re-establishment

Table 8 presented the Statistical analysis from data on plant re-establishment at 20 days after transplanting, which indicated a significant difference ($p<0.01$) among plantlet weaning age. After 20 days of screen house culturing, weaning at 25 days produced the greatest percentage of plant re-establishment.

Between substrate mixtures, there were significant differences ($p<0.01$) were observed. Substrate S1 (soil and cow dung 1:0) recorded the highest plant survival percentage, while S3 (soil and cow dung 1:1) showed the lowest survival percentage.

The age and substrate interaction were also significant ($p<0.01$). The highest plant re-establishment percentage of 98.7 % and 97.8 % were recorded at treatment T1 (Seedlings ages 25 days and cultured on Soil and Cow dung 1:0) and T2 (age 25 days and cultured on Soil and Cow dung 2:1). Next by T3 (Seedlings ages 25 days: on Soil and Cow dung 1:1) with 91.5 % re-establishment. The treatment T6 (Seedlings ages 45 days: on Soil and Cow dung 1:1) observed the lowest with 59.8 % re-establishment after 20 days of culturing.

Plant height

After 15, 40, and 60 days of culturing in vivo, the data collected on plant height indicated significant differences between plantlet age and various substrates. In addition, plantlet age and substrate interaction also were significant (Table 9). The treatment T1 plants showed the greatest plant height with 7.7 cm and 28.3 cm, respectively, from 15 days to 40 days of culturing, but T2 recorded the highest plant height (49 cm) at 60 days of culturing. The lowest was at 60 days of culturing in vivo in T3 and T4 at 15 days, T4, T5, and T6 at 40 days, and T3, T4, and T6 at 60.

Stem diameter

Table 10 presents plant stem diameters recorded after 15, 40, and 60 days of culturing. Significant differences ($p<0.01$) were observed at plantlet weaning age at 15 days and 40 days after transplanting in stem diameters. However, there was no significant difference ($p>0.05$) at 60 days, and the largest plant stem diameters were formed in plantlets weaned at age 25 days, at 15 days, and 40 days after transplanting. Moreover, at 15 days, the substrate showed no significant difference ($p>0.05$) but at 40 days and 60 days after transplanting, highly significant differences ($p<0.01$) were observed. The substrate S3 recorded the largest stem diameters at 40 days and 60 days of culture, joined by the S2 at 60 days. Next, at 40 days and S1 at 60 days of transplanting, the smallest plant stem diameters were formed in substrates S2 and S1.

At 40 days and 60 days of culturing, there were significant differences ($p<0.05$) at 15 days and ($p<0.01$) with interactions between weaning age and substrate mixture. In T1 at 15 days (1.5 mm), in T3 (5.2 mm) and T1 (5.2 mm) at 40 days, and T6, T5, T1, and T3 at 60 days of culturing, respectively with 5.6 mm, 5.6 mm, 4.5 mm and 5.5 mm observed the greatest stem diameter. The lowest diameters at 15, 40, and 60 days respectively, with 1.1 mm, 3.2 mm, and 4.6 mm observed at treatment T4, while at 40 days, with 3.3 mm, was observed at treatment T5.

Table 7. The three Substrates' properties after growing

Substrate	pH in water	pH in KCl	Organic carbon (%)	N total (%)	P assimilable (ppm/100 g)	K assimilable (mg/100 g)
S1	7.24	6.94	0.56	0.01	122.06	45.13
S2	7.38	6.94	1.12	0.04	142.16	62.35
S3	7.53	7.23	1.39	0.05	157.65	84.05

Note: Laboratory SEP-IER, Mali

Table 8. Effect of weaning age of in vitro plantlet and substrate mixture on plant re-establishment percentage at 20 days after transplanting

Age	Substrate (soil and cow dung)			Mean
	1:0	2:1	1:1	(age)
25 days	98.66 [a]	97.77 [a]	91.52 [b]	95.98 a
45 days	83.93 [c]	74.11 [d]	59.82 [e]	72.62 b
Mean (substrate)	91.29 a	85.94 b	75.67 c	
	Age	Substrate	Age*Substrate	
p-value	<.001	<.001	<.001	

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. p-value= probability value

Table 9. Plant height

Treatments		Plant height (cm)		
Age	**Substrate**	**15 days**	**40 days**	**60 days**
25 days	Soil: Cow dung 1:0 = T1	7.69 [a]	28.25 [a]	48.98 [b]
weaning	2:1 = T2	6.67 [b]	21.90 [b]	53.93 [a]
	1:1 = T3	6.04 [c]	22.68 [b]	40.15 [c]
45 days	1:0 = T4	6.10 [c]	15.65 [c]	39.10 [c]
weaning	2:1 = T5	6.67 [b]	14.88 [c]	46.03 [b]
	1:1 = T6	6.57 [b]	15.88 [c]	41.50 [c]
p-value		0.009	<.001	0.001

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. p-value= probability value

Table 10. Plant stem diameter after 15, 40, and 60 days of culturing in vivo

Treatments		Stem diameter (mm)		
Age	**Substrate**	**15 days**	**40 days**	**60 days**
25 days	Soil: Cow dung 1:0 = T1	1.472 [a]	5.18 [ab]	5.46 [ab]
weaning	2:1 = T2	1.277 [b]	4.99 [b]	5.18 [b]
	1:1 = T3	1.20 [bc]	5.24 [a]	5.45 [ab]
45 days	1:0 = T4	1.06 [bc]	3.15 [d]	4.55 [c]
weaning	2:1 = T5	1.11 [bc]	3.33 [d]	5.56 [a]
	1:1 = T6	1.13 [c]	3.93 [c]	5.57 [a]
p-value		0.026	<.001	<.001

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. p-value= probability value

Table 11. Mean number of leaves of different treatments at 40 days of culturing

Age	**Substrate (soil and cow dung)**			**Mean**
	1:0 (S1)	**2:1 (S2)**	**1:1 (S3)**	**(Age)**
25 days	8.43	9.73	9.35	9.17
45 days	8.40	9.75	9.00	9.05
Mean (substrate)	8.41 c	9.74 a	9.18 b	
	Age	Substrate	Age*Substrate	
p-value	0.410	<.001	0.498	

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. p-value= probability value

Table 12. Plant fresh and dry weights at 75 days of culturing

Age	**Substrate**	**Plant fresh weight (g)**	**Plant fresh weight (g)**
25 days	Soil: Cow dung 1:0 = T1	33.43 [c]	2.55 [a]
weaning	2:1 = T2	37.88 [b]	2.63 [a]
	1:1 = T3	25.75 [d]	1.99 [b]
45 days	1:0 = T4	16.70 [e]	1.38 [c]
weaning	2:1 = T5	37.78 [b]	2.45 [a]
	1:1 = T6	41.98 [a]	2.48 [a]
p-value		<.001	<.001

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. p-value= probability value

Stem diameter

Table 10 presents plant stem diameters recorded after 15, 40, and 60 days of culturing. Significant differences ($p<0.01$) were observed at plantlet weaning age at 15 days and 40 days after transplanting in stem diameters. However, there was no significant difference ($p>0.05$) at 60 days, and the largest plant stem diameters were formed in plantlets weaned at age 25 days, at 15 days, and 40 days after transplanting. Moreover, at 15 days, the substrate showed no significant difference ($p>0.05$) but at 40 days and 60 days after transplanting, highly significant differences ($p<0.01$) were observed. The substrate S3 recorded the largest stem diameters at 40 days and 60 days of culture, joined by the S2 at 60 days. Next, at 40 days and S1 at 60 days of transplanting, the smallest plant stem diameters were formed in substrates S2 and S1.

At 40 days and 60 days of culturing, there were significant differences ($p<0.05$) at 15 days and ($p<0.01$) with interactions between weaning age and substrate mixture. In T1 at 15 days (1.5 mm), in T3 (5.2 mm) and T1 (5.2 mm) at 40 days, and T6, T5, T1, and T3 at 60 days of culturing, respectively with 5.6 mm, 5.6 mm, 4.5 mm and 5.5 mm observed the greatest stem diameter. The lowest diameters at 15, 40, and 60 days respectively, with 1.1 mm, 3.2 mm, and 4.6 mm observed at treatment T4, while at 40 days, with 3.3 mm, was observed at treatment T5.

Number of leaves at 40 days of culturing

Table 11 presented the statistical analysis of the data on plant leaf numbers at 40 days of culture. Among in vitro plantlet weaning age means, there was no significant difference ($P>0.05$), while between substrate means indicated highly significant differences ($P<0.01$). The lowest in S1 (Soil and Cow dung 1:0) with 8.41 leaves, and the substrate S2 (Soil and Cow dung 2:1) showed the highest number of leaves (9.74). In the interactions between weaning age and substrate mixture means, no significant differences ($P>0.05$) were observed.

Plant fresh and dry weights at harvest

Table 12 presented the data analysis of plant fresh and dry weights at 75 days of culturing. No significant difference ($p>0.05$) was shown among plantlet weaning age in the fresh plant weight, but between the plant dry weight means there was a highly significant difference ($p<0.01$). The greatest plant dry weight was recorded by the weaning age of 25 days. For both fresh and dry weights, highly significant differences ($p<0.01$) were observed in the means of substrate mixture. The lowest in S1 (Soil and Cow dung 1:0) and the greatest plant fresh and dry weights were both recorded in substrate S2 (Soil and Cow dung 2:1). In the interactions between weaning age and substrate mixture, there were Highly significant differences ($p<0.01$). The highest plant fresh weight was observed in T6, with a mean of 42 g, next by T2 (37.9 mg) and T5 (37.8 mg). Furthermore, the greatest plant dry weights were obtained in T2 (2.6 g), T1 (2.6 g), T6 (2.5 g), and T5, with a mean of 2.5 g, next by T3 (2 g). T4 produced the lowest plant fresh and dry weights, with 16.7 g and 1.4 g, respectively.

Tuber size grading quality

Tables 16 and 17 present an analysis of the data on tuber size grading (A: tubers whose diameter is between 28 and 45 mm, B: tubers with a diameter of less than 28 mm, and C: tubers with a diameter greater than 45 mm). Between the weaning ages of in vitro plantlets for A, B, and C size grading, there were significant differences ($p<0.01$). Seedlings weaned for 25 days produced the highest percentage of tubers in grade A and C sizes than those weaned for 45 days, with the highest percentage in grade B, but on the substrate for grades A, B, and C, significant differences were observed ($p<0.05$). The substrate S1 (Soil and Cow dung 1:0) and S3 (Soil and Cow dung 1:1) produced the highest percentages of grade A tubers. The highest grade B tubers were produced on substrate S2 (Soil and Cow dung 2:1), while substrate S1 produced more tubers of grade C size than the others. Furthermore, between age and substrate ($p<0.01$) for grades A and B, there were significant differences in the interactions; and also significant differences ($p<0.05$) for grade C sizes. The highest percentage of tuber grade A recorded on treatment T1, while the treatments T4, T5, and T6 produced more grade B tubers than the others. On the other hand, in grade C, the treatment T1 mean was significantly higher (Figure 8).

Tubers weight loss in storage

Data recorded on tubers stored for eight weeks on weight loss (%) showed no significant difference ($p>0.05$) for ages 2, 4, 6, and 8 weeks of storage. There was no significant difference for substrate at 4 weeks ($p>0.05$), but significant differences occurred for substrate at 2, 6, and 8 weeks of storage ($p< 0.05$). From 2 to 8 weeks of storage, the interactions (age*substrate) were observed to be significant ($p<0.01$). The lowest weight loss percentages in stored tubers were obtained in substrate S1 and the highest in substrates S2 and S3. At two weeks, the lowest weight loss percentages were observed in the tubers in treatments T1, T2, T6, and T4. T1 and T6 at four weeks, T1 at six weeks, and T1 and T6 at eight weeks of storage. The highest was shown by treatments T3 at two weeks, T4 and T5 at four weeks, T5 at six weeks, and T2 tubers at eight weeks of storage (Table 16).

Tuber sprout numbers after three months in storage

The potato-tuber sprout number results after three months in storage are presented in Table 17, Figures 9 and 10. Significant differences ($p< 0.01$) occurred for the weaning age of in vitro plantlets. The lowest tuber sprout number was observed on plantlets weaned for 45 days, while plantlets at 25 days recorded the highest number of sprouts.

For the substrate used for transplantation, significant differences ($p<0.01$) were observed. The number of sprouts on a tuber on substrates S2 (soil and cow dung 2:1) and S3 (soil and cow dung 1:1) significantly reduced than to S1 (soil and cow dung 1:0), which produced the highest number of sprouts on a tuber. On Age and Substrate interaction, no significant difference ($p>0.05$) was observed.

Table 13. The yield of tubers in tons per hectare

Age	Substrate (soil and cow dung)			Mean (age)
	1:0 (S1)	2:1 (S2)	1:1(S3)	
25 days weaning	58.12 [a]	59.28 [a]	44.03 [c]	58.81 a
45 days weaning	33.08 [d]	47.08 [b]	46.03 [bc]	42.06 b
Mean (Substrate)	45.60 b	58.18 a	45.03 b	
	Age	Substrate	Age*Substrate	
p-value	<.001	<.001	<.001	

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. p-value= probability value

Table 14. The average weight of one tuber

Age	Substrate (soil and cow dung)			Mean (age)
	1:0 (S1)	2:1 (S2)	1:1(S3)	
25 days weaning	11.98 [a]	7.83 [b]	7.25 [c]	9.02 a
45 days weaning	5.56 [e]	5.54 [e]	6.14 [d]	5.75 b
Mean (substrate)	8.77 a	6.69 b	6.69 b	
	Age	Substrate	Age*Substrate	
p-value	<.001	<.001	<.001	

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. p-value= probability value

Table 15. Number of tuber per plant at harvest

Age	Substrate (soil and cow dung)			Mean (age)
	1:0 (S1)	2:1 (S2)	1:1(S3)	
25 days weaning	4.85 [d]	7.58 [b]	6.08 [c]	6.17 b
45 days weaning	5.95 [c]	8.50 [a]	7.50 [b]	7.32 a
Mean (Substrate)	5.40 c	8.04 a	6.79 b	
	Age	Substrate	Age*Substrate	
p-value	<.001	<.001	0.009	

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. p-value= probability value

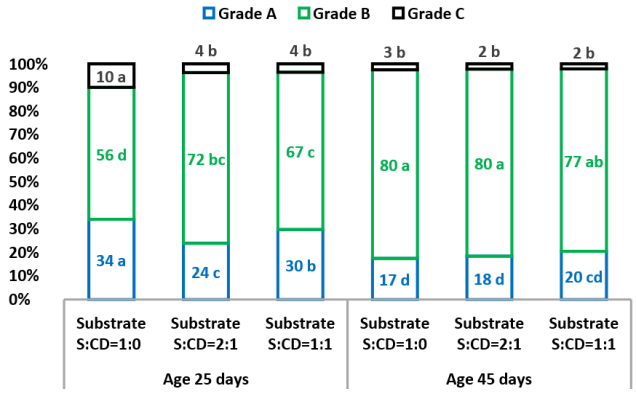

Figure 8. Tuber size grading. Letters represent significant differences among means according to Fisher's LSD test at 5% significance. Grade A (p-value: <.001), Grade B (p-value: 0.001), Grade C (p-value: 0.043)

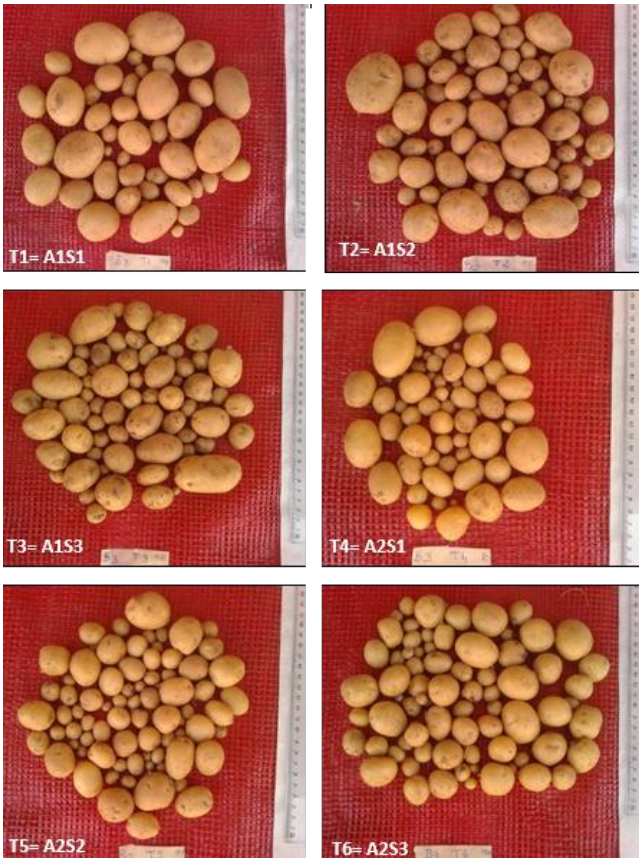

Figure 9. Tubers produced at harvest by 10 plants under the 6 treatment conditions. A1= plantlet weaning age 25 days; A2= plantlet weaning age 45 days. S1= soil and cow dung 1:0; S2= soil and cow dung 2:1 and S3= soil and cow dung 1:1

Table 16. Tubers' percentage weight loss from harvest to 8 weeks in storage

Treatments		Tubers weight loss (%)			
Age	**Substrate**	**2 WIS**	**4 WIS**	**6 WIS**	**8 WIS**
25 days	Soil: Cow dung 1:0 = T1	4.46^{a}	6.76^{a}	9.37^{a}	12.50^{a}
weaning	2:1 = T2	4.84^{a}	7.46^{b}	10.87^{bc}	18.48^{d}
	1:1 = T3	6.50^{c}	8.50^{c}	12.54^{d}	17.75^{cd}
45 days	1:0 = T4	5.05^{ab}	8.26 ^{c}	10.73^{bc}	15.08^{b}
weaning	2:1 = T5	5.53^{b}	8.27^{c}	11.58^{c}	15.86^{bc}
	1:1 = T6	4.43^{a}	7.07^{ab}	10.31^{b}	13.60^{ab}
p-value		<.001	<.001	<.001	0.003

Note: Letters represent significant differences among treatments according to Fisher's LSD test at 5% significance. The comparison's direction is ascending. WIS: week in storage. p-value= probability value

Table 17. Tuber sprout number after 3 months in storage

Age	Substrate (soil and cow dung)			Mean (age)
	1:0 (S1)	2:1 (S2)	1:1(S3)	
25 days weaning	9.45	7.72	7.40	8.19 b
45 days weaning	6.92	6.50	5.75	6.39 a
Mean (Substrate)	8.19 b	7.11 a	6.58 a	
	Age	Substrate	Age*Substrate	
p-value	<.001	0.004	0.287	

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. The comparison's direction is ascending. p-value= probability value

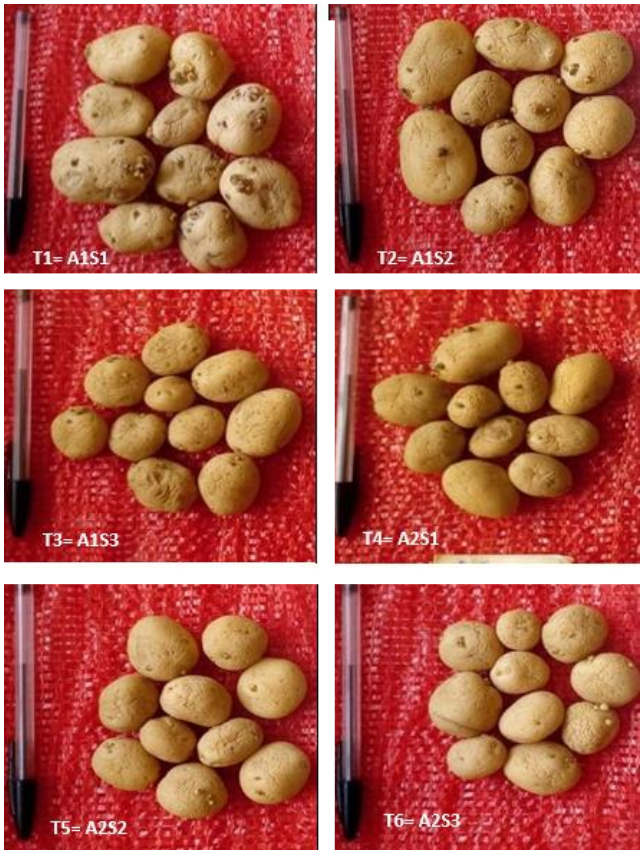

Figure 10. Tubers produced under the 6 treatment conditions 3 months after storage. A1= plantlet weaning age 25 days; A2= plantlet weaning age 45 days. S1= soil and cow dung 1:0; S2= soil and cow dung 2:1 and S3= soil and cow dung 1:1

Table 18. Sprout numbers per eye after 3 months of storage

Age	Substrate (soil and cow dung)			Mean (age)
	1:0 (S1)	2:1 (S2)	1:1(S3)	
25 days weaning	1.90 ^{b}	1.42 ^{a}	1.35 ^{a}	1.56 b
45 days weaning	1.31 ^{a}	1.20 ^{a}	1.25 ^{a}	1.25 a
Mean (Substrate)	1.60 b	1.31 a	1.30 a	
	Age	Substrate	Age*Substrate	
p-value	0.001	0.008	0.047	

Note: Letters represent significant differences among means according to Fisher's LSD test at 5% significance. The comparison's direction is ascending. p-value= probability value

Sprout number per eye after three months of storage

Table 18. shows the effect of the weaning age of substrate and in vitro plantlet for transplanting on sprout numbers per eye after three months of storage. There was a significant difference (P<0.05) among the means for weaning age. The lowest number of sprouts per eye on tubers grew at a weaning age of 45 days and the highest in tubers from 25 days.

Furthermore, depending on the substrate used for transplanting, significant differences (p< 0.05) were observed g. The tubers with the lowest sprouts per eye produced on substrates S2 (soil and cow dung 2:1) and S3 (soil and cow dung 1:1) and the highest in tubers on S1. Also, for age and substrate interaction, there were

significant differences ($p<0.05$). The lowest number of sprouts per eye was shown by treatments T2, T3, T4, T5, and T6, in which only T1 showed the highest.

Discussion

Effects of culture media on plants grown in vitro

The study showed that after 30 days of in vitro, culturing growth and development parameters stimulated by the culture medium M7, 40 mL of coconut water, and 250 mg of potassium nitrate per liter of MS medium were used. This stimulation may be due to adequate quantities of coconut water and potassium nitrate. Coconut water contains several organic compounds such as sucrose; vitamins of B group: nicotinic acid, biotin, and folic acid; phytohormones: auxin, cytokinins, gibberellins, and abscisic acid; and mineral nutrients: potassium, sodium, calcium, phosphorous (Yong et al. 2009; Mullukattil 2013; Reddy and Lakshmi 2014). The in vitro explants growth could be stimulated on a medium supplemented with 5% to 15% coconut water (CIDES 1999; Prades et al. 2011), which is the quantity of 5% close to the concentration of 40 mL of coconut water per liter medium (4% of culture medium volume). The coconut water at 40 mL combined with 250 mg of potassium nitrate per liter MS medium played a role in the plant growth stimulation observed.

The media M7 (MS + 40 mL/L of coconut water + 250 mg of potassium nitrate), M8 (MS + 40 mL/L coconut water +1,000 mg of potassium nitrate), M10 (MS+100 mL/L coconut water + 1,000 mg of potassium nitrate) and M11 (MS+300 mL/L coconut water +250 mg of potassium nitrate) observed to promote faster shoots emergence than the other media. Those media resulted in 100 % emergence, compared to M1 (MS medium, the control), which recorded shoot emergence at 83 %.

Furthermore, after 30 days of culturing, the greatest in vitro plantlet height (16 cm) was shown by the culture medium M7 (MS + 40 mL/L of coconut water + 250 mg of potassium nitrate) than the MS medium used as the control, with a maximum of 10 cm shoot length. The adequate quantity of growth-promoting substances may explain that in coconut water, such as N6-Furfuryladenine (Kinetin), which promotes cell division in plants (Yong et al. 2009) and the effect on the cultured explants of the nitrogen contained in potassium nitrate. Nitrogen is an essential element for photosynthesis (chlorophyll) and cell formation. Therefore, the main factor of plant growth and quality influences plants' protein content (UNIFA 2005).

After 30 days of in vitro culturing, the highest number of nodes (11.5) was observed in culture medium M7 as a supplement to MS medium containing 40 mL of coconut water and 250 mg of potassium nitrate per liter. However, M1 (MS), the control medium, recorded only seven nodes. The number of nodes per plant refers to the plant multiplication rate. The culture medium M7 recorded the highest mean number of leaves (13.5). But the control, M1 (MS), recorded fewer leaves than M7 containing 40 mL/L of coconut water, and 250 mg/L of potassium nitrate were used as supplements to the MS medium. Another study stated that cytokinin (kinetin) promotes bud formation in many in vitro cultured organs, such as leaves and node numbers reported by Afshin et al. (2011).

The root number per plant was greatest in M7 and M8 (9 roots) versus M1 (MS), which recorded 5.1 roots, which may be due to the coconut water's indole- 3-acetic acid (auxin). The IAA plays a significant role in growth regulation and plant root emergence (Muhammad et al. 2015). Even though this study realized that M6, M11, and M12, 300 mL of coconut water per liter, revealed the least root numbers per plant, 1.6, 2.2, and 1.2 roots, respectively. The higher cytokinin concentration can explain that in 300 mL of coconut water per liter. The CIDES (1999) reported that cytokinin at higher concentrations in a culture medium is responsible for multiple shoots and callus formation.

The highest plantlet fresh and dry weights (545.5 and 36.4 mg) after 30 days after propagating in vitro were observed in culture medium M7 (MS + 40 mL/L of coconut water and 250 mg of potassium nitrate). Moreover, 50 mL/L of coconut water in a culture medium in Calanthe hybrids increased plant fresh and dry weights (Abdullahil et al. 2011). In this study, the maximum fresh and dry potato plantlets were in M7, where MS was supplemented with 40 mL/L of coconut water and 250 mg of potassium nitrate. This coconut water concentration is comparable to the 50 mL/L cited before.

The lower concentrations of coconut water (40 mL) and potassium nitrate (250 mg) per liter of MS medium interaction after 30 days of culturing have significantly affected all the in vitro growth parameters. Conversely, plant growth in height and root emergence were blocked by the higher concentrations of coconut water (300 mL per liter of MS medium).

Physiological age and substrate effects on mini tubers production in vivo

The pH in the experiment was reduced on the three substrates used compared to the soil's initial state before the substrates' mixture and planting. Applying cow dung or mineral fertilizer (NPK) to all three substrates could decrease pH levels in all substrates. This result is consistent with Monirul et al. (2013) and Suh et al. (2015). However, the reduction was shown more with the substrate S1 (soil and cow dung 1:0), with no cow dung but had the application of mineral fertilizer (1,000 kg/ha of NPK 17:17:17), with a reduction of 5.7% of the observed initial pH (7.68). The treatments T1 (age 25 days and substrate-only soil) and T2 (age 25 days and substrate 2:1 Soil and Cow dung) recorded the maximum plant re-establishment percentage.

The growing substrate pH may affect plantlets' in vivo survival and growth (Conner and Thomas 1982). The laboratory analysis showed that the maximum plant survival percentage was observed at the lower pH in substrate S1 (7.24). On the other hand, S2 (soil and cow dung 2:1) promoted significantly higher plant stem length, stem diameter, and fresh and dry biomass formation. Moreover, the effect of plantlet weaning age was insignificant for plant stem diameter and fresh and dry weight. The highest yield of tubers was recorded on the treatments T2 (59.28 t/ha) and T1 (58.12 t/ha). That is

possibly due to the level of organic carbon and the pH in S2. However, S3 presented a higher amount of organic carbon compared to S2 but showed a higher level of alkaline pH than others, which could be a disadvantage for tuber formation and plant growth. According to Mimouni (2011), the soil's alkaline pH can block the absorption of phosphorus, copper, iron, manganese, boron, and zinc.

Furthermore, in treatment T5 (weaning age 45 days and substrate 2:1 Soil and Cow dung) have the maximum number of tubers per plant, which may be due to the ability to increase nutrient availability through high biological activity and the high nutrient level of the two substrates (Pengthamkeeratia 2011). Plantlets with a weaning age of 25 days produced significant numbers of grade A, while the substrates S1 and S3 (soil and cow dung 1:1) means were significant in grade A.

The substrate composition positively affected the weight loss (%) of stored tubers after eight weeks, and the lowest percentages were obtained in substrates without cow dung. The Substrate S1 (only soil) showed the same trend of tubers' low weight loss from the 2nd week to the 8th week of storage, and the highest weight loss percentages in stored tubers were obtained in S2 (Soil and Cow dung 2:1), and S3 (soil and cow dung 1:1) that the substrate contained cow dung. The highest percentages of weight loss in Substrates S2 (Soil and Cow dung 2:1) and S3 (soil and cow dung 1:1) can be the lower immaturity of tubers' skin. Moreover, at harvest time was observed in Substrate S1 (only soil) plots, all plants had reached the senescence stage (physiological maturity). Generally, the senescence stage progressively changes the leaves' color to yellow from the base to the top, with subsequent drying out. Even though plants in all plots were still growing in substrates S2 (Soil and Cow dung 2:1) and S3 (soil and cow dung 1:1), that can lead to immature tubers harvested from these treatment plots. A study by Paris IV University in collaboration with ARVALIS showed that water loss in tubers was proportional to Vapor Pressure Deficit (VPD) between the tuber and the ambient air and also showed a significantly higher loss in case of the immaturity of the skin or injury of tubers (Martin 2006).

Furthermore, for the weaning age of 45 days at the substrates S2 (soil and cow dung 2:1) and S3 (soil and cow dung 1:1), the number of sprouts per tuber and sprouts number per eye on tubers was minimized significantly. These results differ from the weaning age of 25 days and substrate S1 (soil and cow dung 1:0) after three months of storage, which promoted early emergence and higher sprouts number on the tuber. One of the most important factors in the deterioration of quality during storage is tuber sprouting (ARVALIS /Institut-du-végétal 2013). The tuber dormancy time for the small size grade is longer than that of larger sizes reported by Reust (1982). Moreover, the weaning age of 45 days and the substrates S2 (soil and cow dung 2:1) conditions that the maximum quantities of small-size grade tubers were produced, revealed in this current study.

In conclusion, the study's results showed the highest plantlets multiplication rate on the culture medium M7 (MS+40 mL/L of coconut water +250 mg of potassium nitrate) after 30 days of in vitro culture, with an average number of 11.45 nodes per plant. Furthermore, on all the in vitro growth parameters, the interaction between lower concentrations of coconut water (40 mL) and potassium nitrate (250 mg) per liter of MS medium has significantly positive effects. Also, during 30 days of culturing, the higher concentrations of coconut water (300 mL) potassium nitrate (1,000 mg) per liter of MS medium adversely affect plant growth parameters. Moreover, at 20 days after transplanting in vivo, the Plantlet weaning age of 25 days and the post-flask culture substrate S1 (only soil) appears to give the best plant survival percentage. On all plant in vivo growth and tuber yield parameters, substrate S2 (soil and cow dung 2:1) was positively affected. Finally, in storage three months after harvest, the weaning age of 45 days and the substrates S2 (soil and cow dung 2:1) and S3 (soil and cow dung 1:1) conditions produced significantly reduced mini tubers quality loss.

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