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Phyto-Saponins as a Natural Adjuvant for Delivery of Agromaterials through Plant Cuticle Membranes

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With growing use of synthetic adjuvants in modern agriculture, their impacts on the environment are being questioned. In a search for an environmentally safe phyto-adjuvant, we have investigated natural glycosidic saponin for delivery of agromaterials through plant cuticle membranes. Four saponin preparations from *Quillaja saponaria* bark (QE), obtained from Sigma-Aldrich, and *Balanites aegyptiaca* fruit mesocarp (ME), kernel (KE), and root (RE), isolated and characterized in our laboratory, were used for testing the delivery of [^{14}C]-2,4-dichlorophenoxyacetic acid (2,4-D) across isolated intact astomatous adaxial *Citrus grandis* leaf cuticle membranes (CMs). The results showed that both *Q. saponaria* and *B. aegyptiaca* saponin preparations enhanced delivery of 2,4-D through CMs. Among the saponin preparations, ME exhibited a significantly higher level of delivery of 2,4-D with a concentration effect (2% being the highest). Transmission electron microscope (TEM) and dynamic light scattering (DLS) characterization of these saponin preparations in aqueous solution clearly demonstrated the formation of nanoscale vesicles. Various possibilities for a natural amphiphatic phyto-saponin as a delivery adjuvant through CMs are discussed.

KEYWORDS: Phyto-saponins; cuticle membrane; phytoadjuvant; nanovesicles; *Balanites aegyptiaca*; *Quillaja saponaria*

INTRODUCTION

Plant cuticle is a thin (<0.1−10 μm) continuous layer or membrane of predominantly lipid material that covers the entire external surface of plants, including leaves of the higher plants, and forms the interface between the foliage and the atmosphere (*1*). The main function of the plant cuticle is to minimize water loss from plants when stomata are closed and to protect the plant against physical, chemical, and biological attack; however, cuticle remains the main barrier to the penetration of compounds applied to the foliage or ground (*2*). The outer surface of the cuticle is covered by epicuticular wax which can occur in many forms, from amorphous to crystalline deposits, and consists of complex mixtures of long-chain aliphatic and cyclic components (*3*). Plant cuticles also contain non-lipid constituents such as polysaccharides and phenolics. It has been reported that various phytosterols such as pentacylic triterpenols and Δ^5-sterols are found in the cuticular membrane in leaves and rhizomes (*4*). The epicuticular wax layer of the cuticle is the main barrier to penetration of applied material because of its hydrophobic nature; this layer must be penetrated before any applied agromaterial can enter living cells.

In the past few decades, the use of agricultural adjuvants and/or surfactants has become common practice in foliar application to enhance the delivery of agromaterials to the inner tissue of the plant through the cuticular layer (*5*). Although the term "adjuvant" has numerous meanings and has been the source of unending confusion, it is commonly understood that agricultural adjuvants are any materials other than water that are added to the agromaterials to increase their efficiency when applied to the plants (*6*). The major category of agricultural adjuvants is the oil-based group. Historically, these have been of mineral origin, but these are gradually being superseded by vegetable oils and their derivatives. The second major category of adjuvants consists of surfactants (surface-active agents). These include a wide range of materials, which can be organic or inorganic in nature. Other chemical categories include various polymers, film-forming materials, and inorganic salts (*7*). Among the surfactants, silicone-based nonionic surfactants are the most commonly recommended and used adjuvants. Although these surfactant-type adjuvants increase the diffusive mobility of agromaterials across the cuticle, thereby increasing the penetration potential, since these adjuvants were originally designed for herbicides, severe necrotic damage to the treated leaves is commonly encountered when using these surfactants with foliar nutrients (*8*).

Although adjuvants are generally considered "inert" or essentially nonhazardous, the main reason behind nondisclosure of most ingredients is the manufacturers' reticence (*9*). The long-term fates of most adjuvants in soils and elsewhere in the environment are also largely unknown because of the lack of

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long-term monitoring data; however, environmental questions have recently been raised about these products. The practice of foliar application of nutrients, growth regulators, pesticides, and herbicides has grown significantly in recent years; however, often the results of plant application of agromaterials are insufficient. Thus, considerable technical, economic, and ecological interests are invested in increasing the efficacy of these applications. The efficacy of foliage-applied agrochemicals depends on the amount of active ingredients (a.i.) delivered into the target species and/or tissues across the cuticle layer which forms a natural interface between the plant and the environment. To improve the uptake of such agromaterials, a wide range of surfactants and adjuvants have been developed and tested, and various delivery adjuvant mechanisms have been recommended over the past 30 years (*8*). Most of these delivery adjuvant systems are based on either partially or completely synthetic materials, raising environmental questions. Traditional amine ethoxylate surfactants are now less preferred due to unfavorable toxicity profiles of aquatic fauna, while nonylphenol ethoxylates have recently been under public scrutiny for reported estrogenic side effects. The trend within the oil-type adjuvants is likely to continue to move toward vegetable oils and their derivatives due to poor biodegradation of mineral oils (*10*).

Saponins, which are high-molecular weight glycoside compounds, consist of both lipophilic and hydrophilic moieties and have been used as an adjuvant in veterinary medicines to promote penetration of drugs through the stratum corneum (SC), the major barrier layer in the skin; interest in using the saponin-based adjuvants in humans is also constantly growing (*11*). The immunological role and divergent biological activities, in the presence of both hydrophilic and lipophilic moieties, have made glycosidic saponins the best adjuvant for drug delivery (*12*). Although various saponin-rich extracts have commonly been used in agriculture for their different activities, there has been no report about the use of these extracts as an agricultural adjuvant. Considering the use of saponin in vaccine delivery and the protective activities of saponins, we surmised that amphiphilic saponin may also be used as a nonionic, environmentally safe bioadjuvant for foliar application of agrochemicals.

Considerable research has been focused in recent years on the use of vesicles, mainly liposomes, for delivery of drugs and active substances through biological membranes, especially in the medical field (*13*). Formation of micelle-type vesicles from saponin compounds has been reported in the literature in relation to triterpenoid saponins (*14*). However, no report has mentioned either the vesicle production from steroidal saponins or its correlation with adjuvant delivery across plant membranes. Therefore, it is crucial to characterize the production of vesicles from such saponins and their association with adjuvant delivery through plant membrane to broaden the understanding of the use of saponins.

As a model test system, we have chosen the most common saponin used in veterinary medicines, *Quillaja saponaria* extract, and extracts of *Balanites aegyptiaca*. *Q. saponaria* is a large evergreen tree with shiny, leathery leaves and thick bark, which is native to Peru and the arid region of Chile, with triterpenoid saponin as the main bioactive compound (*15*), while *B. aegyptiaca* is a desert plant belonging to the family Zygophyllaceae found in Africa, south Asia, and the Arabian Peninsula, with steroidal saponin as the main bioactive compound in its plant tissues (*16*, *17*). Our study has shown that extracts of the *Balanites* plant contain a large quantity of easily extractable saponins and demonstrate strong growth inhibition against some economically important phytofungi in vitro (*18*). Although *B. aegyptiaca* is commonly grown in the dry arid regions of most of Africa and Asia, this plant has not been domesticated and is considered one of the most neglected plant species of the region (*19*). Any plant-based uses of the plant would help in its domestication.

Since saponins are fairly safe and easily biodegradable natural products, a saponin-based delivery system may provide an answer to the environmental concerns raised by the use of synthetic adjuvants. The aim of this study was to investigate an environmentally friendly bioadjuvant for the delivery of agromaterials through the plant cuticular barrier. Specific objectives included qualitative and quantitative characterization of the main compounds in the various saponin preparations, evaluation of the effect of saponin preparations on the rate of delivery of 2,4-dichlorophenoxyacetic acid (2,4-D) through the isolated plant cuticle membrane, and microscopic characterization of assembly of saponin preparations in aqueous solution.

MATERIALS AND METHODS

Preparation of the Saponin Extracts. The *Quillaja* saponin preparation (QE) was made by diluting a commercial saponin extract of *Q. saponaria* bark purchased from Sigma (Sigma-Aldrich). Three *Balanites* saponin preparations were made by diluting the laboratory-prepared fruit mesocarp extract, kernel extract, and root extract of *B. aegyptiaca*. The fruits and roots of *B. aegyptiaca* were collected from the *Balanites* orchard located in the Kibbutz Samar of southern Israel and authenticated by U. Plitman from the herbarium in the Hebrew University of Jerusalem (Jerusalem, Israel). Voucher specimen (76816) was deposited in the herbarium of the Hebrew University of Jerusalem. Methanol extracts of *Balanites* fruit mesocarp, kernel, and roots were prepared in our laboratory as described elsewhere (*20*). In brief, the mesocarps of the fruits were scraped, lyophilized, and then ground. The ground mesocarp was defatted with petroleum ether (bp 60−80 °C) and extracted with methanol. The methanol was evaporated under vacuum, and a brown precipitate (residual extracts) was obtained. The kernels of the fruits were ground and defatted (oil extraction) with petroleum ether. The defatted kernel cake was extracted with methanol. After removal of methanol under vacuum, a whitish-brown precipitate was obtained. The roots were first cut into small pieces, dried in an oven (<70 °C), and ground. After the samples had been ground, the petroleum ether-defattd material was extracted with methanol as described for mesocarp and kernel preparations. The concentrations of saponins in all three extracts were enriched by removing the free sugar residues from the extracts using a solid-phase extraction (SPE) procedure as described by Suzuki et al. (*21*). The methanol extracts of fruit mesocarp, kernel, and root of *B. aegyptiaca* were dissolved in water and loaded into a 35 mL, 10 g, C18 SPE extraction cartridge (Waters, Milford, MA). The SPE cartridge was initially washed with 2 column volumes of DDW and than with 35% methanol. The saponins were eluted from the cartridge with 2 column volumes of 100% methanol. These methanol preparations were dried under vacuum and named ME (mesocarp extract), KE (kernel extract), and RE (root extract).

Characterization of Saponins. Qualitative and quantitative characterization of *B. aegyptiaca* preparation (ME, KE, and RE) was performed using high-performance liquid chromatography and mass spectrometry (LC−MS) for saponin compounds. For this study, a Waters 2690 HPLC system equipped with an Agilent (Palo Alto, CA) RI (refractive index) detector with a 70:30 MeOH/H_2O mobile phase at a flow rate of 0.2 mL/min was operated at room temperature. A single-ion-trap mass spectrometer (Esquire 3000 Plus, Bruker Daltonik), equipped with an ESI (electrospray ionization) interface as the ion source in negative ion mode, was employed for MS analyses (*18*). The total amount of sapogenin of these saponin preparations was also determined by the spectrophotometric method as described by Baccou et al. (*22*) and Uematsu et al. (*23*), with some modification as described by Chapagain and Wiesman (*20*) as a diosgenin equivalent. The amount of sapogenin (diosgenin) was determined by measuring the absorbance

at 430 nm, based on the color reaction with *p*-anisaldehyde, sulfuric acid, and ethyl acetate.

Leaf Cuticle Isolation. Full-grown matured leaves of *Citrus grandis* L. having astomatous cuticles were collected from the *C. grandis* plant grown in the commercial orchard of the Kibbutz Yad-Mordechai, Israel, and washed in DDW. After disks 20 mm in diameter (using a cork borer) had been punched out of the leaves, the cuticles were isolated enzymatically by incubating the leaf disks in a mixture (1:1) of cellulose 203-13L (Biocatalysts) and Pectinase 62L (Biocatalysts) as described by Schonherr and Riederer (*24*) in 1% (w/w) citric acid buffer (0.1 M) at 40 °C and pH 4. After a few days, astomatous cuticles from the upper leaf epidermis were collected, rinsed extensively, desorbed in DDW, air-dried on Teflon disks, and stored in a refrigerator until they were used. These isolated cuticles are hereafter termed cuticular membrane (CMs).

Delivery Experiments. In the first phase of the study, four donor solutions (1% QE, ME, KE, and RE preparations) were prepared by adding ^{14}C-labeled 2,4-D (specific activity of 19.2 mCi/mmol, Sigma) as a tracer (30000−40000 cpm/μL) and compared with both negative (DDW) and positive (1% Triton X-100, Sigma) controls. Rates of cuticular penetration were measured at 30% relative humidity (RH) and 30 °C using the SOFU procedure as described by Schonherr (*25*) and modified by Wiesman et al. (*8*). A special delivery system with a thermostat desorption chamber and controlled environment was designed for the experiment. CMs were mounted between the lid and bottom of the desorption chambers using silicon grease (Bayer). Each CM was tested for leaks. DDW was added to the desorption chamber for 24 h, after which the DDW was withdrawn and a 10 μL droplet of donor solution (1% solution of QE, ME, KE, RE preparation, and DDW with [^{14}C]-2,4-D) was placed in the center of the CM. After the water evaporated from the donor solution, the chambers were filled again with DDW which served as the receiving solution.

Receiver solution (DDW) was quantitatively withdrawn after 1, 4, 24, 48, 72, 96, 120, 144, and 168 h for scintillation counting and was replaced with a fresh solution. At the end of the experiment, the CM was removed from the chamber and, after the scintillation cocktail was added, counted to determine the amount of radioactive material left on the surface of the CM. A Beckman LS 1701 scintillation counter (Beckman Coulter) was used to determine the radioactivity of the samples. The amount applied (M_0) was calculated by summing the amounts penetrated (M_t) plus the amount left on the CM at the end of the experiment in the individual CMs. Thus, M_t/M_0 is the fraction that penetrated, and $1 - M_t/M_0$ is the fraction remaining on the surface of the CM. Data were plotted as $-\ln(1 - M_t/M_0)$ versus time as described by Schonherr (*25*). The experiment was repeated three times.

In the second phase of the study, six concentrations of ME (0.1, 0.25, 0.5, 1.0, 2.0, and 5%, w/v) were tested as donor solutions by adding ^{14}C-labeled 2,4-D and compared with the negative control (DDW). The preparation of both donor and receiver solutions and the experimental methods were the same as in the first phase of the study. In this phase of study, the experiment was also repeated three times.

In the third phase of the study, the effect of the penetration of 1% ME was compared at different humidity levels (30, 60, and 90% RH) and temperatures (30, 45, and 60 °C). While humidities were being compared, the temperature was set at 30 °C; during the temperature study, the relative humidity was set at 30%. In all experiment, at least 20 CMs were used in each treatment.

Microscopic Study. Scanning electron microscopy (SEM) characterization of isolated astomatous adaxial cuticle membranes was carried out using a Quanta 200, FEI SEM (Japan) device equipped with a back-scattering detector. Scanning electron images of the outer and inner surface of the CMs, used for the delivery experiments, were taken.

Transmission electron microscopy (TEM) characterization of the different saponin preparations was carried out on a JEOL-JEM-1230 electron microscope (Japan) using a negative staining technique, employing a saturated uranyl acetate solution, using Ultrapure water (Biological Industries). The grid (300 mesh copper Formvar/carbon) was immersed in a 1.0% solution of each ME, RE, KE, and QE preparation for 1.5 min and then stained with the uranyl acetate solution for 1.5 min. The grid was then dried at room temperature on Whatmann filter paper (*26*). The dried grids were examined at 8000 kV, an accelerating voltage, and 25000× magnification.

Table 1. Major Saponins and Their Proportional Amount (percent of total saponins) of the *B. aegyptiaca* Saponin Preparations Used in This Study[a]

sapoinn molecular mass[b] (Da)	percent of total[c]		
	ME	KE	RE
1046	3.1 ± 0.3	7.1 ± 0.5	–
1064	42.3 ± 3.1	7.1 ± 0.4	4.4 ± 0.4
1078	24.6 ± 2.1	29.5 ± 1.2	–
1196	3.1 ± 0.5	1.1 ± 0.3	52.7 ± 2.8
1210	26.6 ± 1.8	36.0 ± 4.3	2.0 ± 0.3
1224	–	19.0 ± 0.9	32.0 ± 1.2
1340	–	–	2.1 ± 0.3
1516	–	–	1.5 ± 0.2
1530	–	–	2.6 ± 0.4
1572	–	–	1.0 ± 0.1
1586	–	–	1.8 ± 0.2

[a] ME, KE, and RE are the saponin preparations obtained from the fruit mesocarp, kernel, and roots of *B. aegyptiaca*, respectively. [b] The molecular mass of the major saponin was determined by ESI-MS. [c] Each value is the mean of three values ± the standard error, quantitatively derived from the peak area of the saponin chromatograms obtained by HPLC-RI.

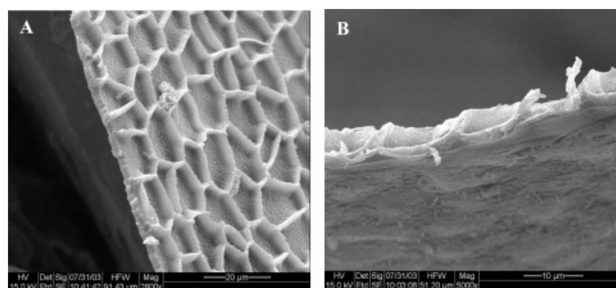

Figure 1. Scanning electron micrograph of an isolated leaf cuticular membrane: (**A**) inner surface and (**B**) outer surface.

Vesicle Size Analysis. Vesicles formed by the various saponin preparations were assessed using dynamic light scattering measurement techniques on an ALV-NIBS high-performance particle sizer. Each 1% saponin preparation (ME, KE, RE, and QE) was prepared using Ultrapure water (Biological Industries) under dust-free conditions. Measurements were performed at a 173° angle at 632 nm and 25 °C. The light source was an argon ion laser, and the photoelectron count-time autocorrelation function was calculated with a BI2030AT (Brookhaven Instruments) digital correlator and analyzer, using the method of cumulants or the constrained regularization algorithm CONTIN applying the Stokes−Einstein relationship to the translational diffusion coefficients, providing an intensity-weighted distribution of hydrodynamic sizes (*27*).

Statistical Analysis. Statistical analysis of the data was performed with JMP (version 4, SAS Institute, Inc., Cary, NC) using the Tukey−Kramer HSD test for determining the significant difference among treatments at the $P = 0.05$ level of significance.

RESULTS

Characterization of Saponin Preparations. The LC−MS experiments showed that there were five saponins with molecular masses of 1046, 1078, 1064, 1196, and 1210 Da in the ME preparation. Among them, saponin with a molecular mass of 1064 Da (ca. 43.2% of total saponins) was the main one (**Table 1**). MS/MS analysis of this compound demonstrated a clear phenomenon of a steroid saponin with a diosgenin aglycon (**Figure 2**) that linked to a single glucose unit in position 26, and a glucose−glucose−rhamanose chain attached to position 3 and hydroxy-linked to position 22 as described by Lindin (*28*).

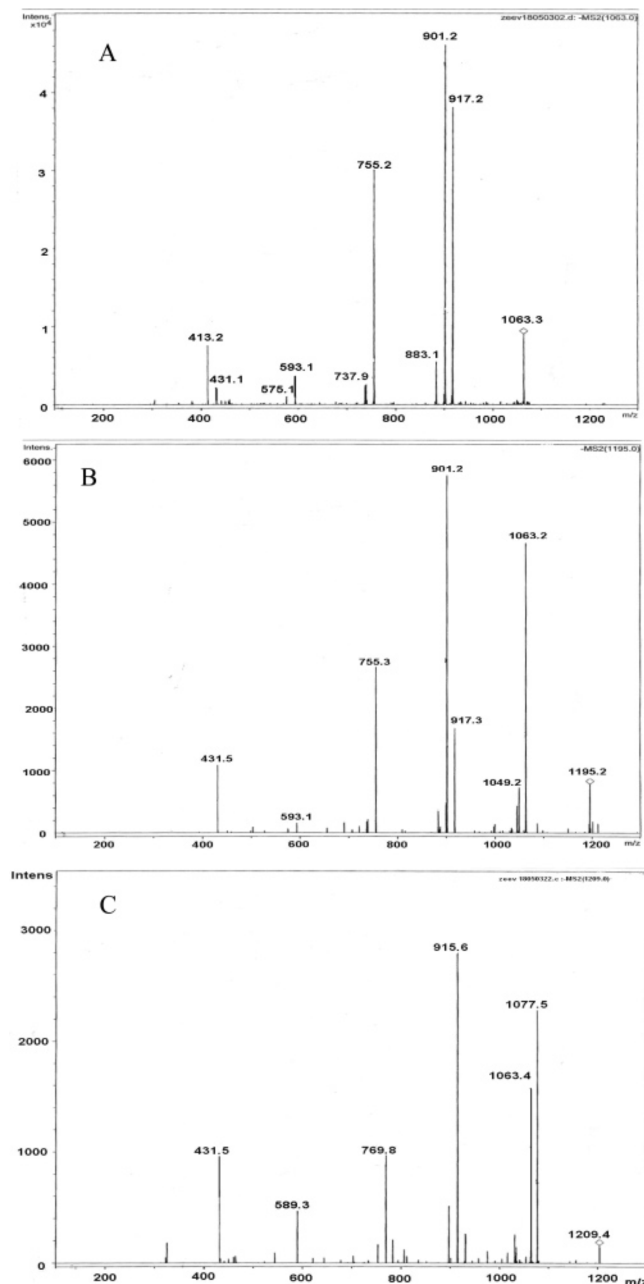

Figure 2. MS/MS spectra of the main saponins of *B. aegyptiaca* plant tissue in negative ion mode: (**A**) fruit mesocarp extract (ME), (**B**) kernel extract (KE), and (**C**) root extract (RE).

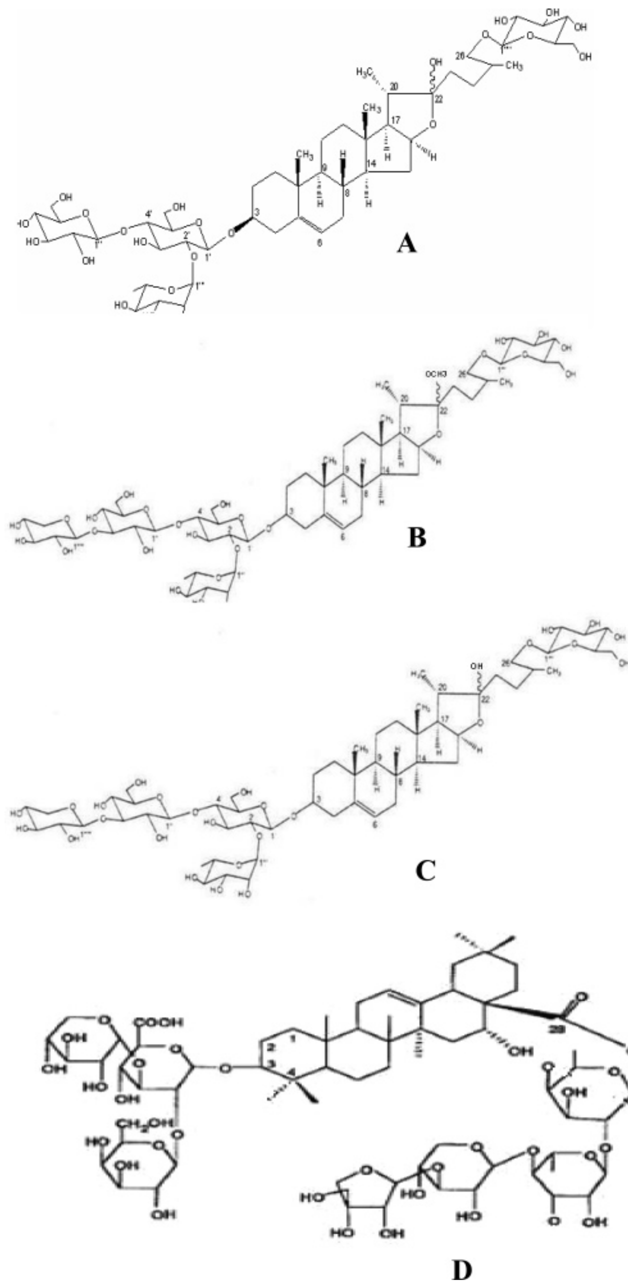

Figure 3. Structure of the major saponin of the *B. aegyptiaca* and *Q. saponaria* plant tissues: (**A**) fruit mesocarp extract (ME), (**B**) kernel extract (KE), and (**C**) root extract (RE) of *B. aegyptiaca* (*28*) and (**D**) plant extract of *Q. saponaria* (QE) (*29*).

The structure of this compound is presented in **Figure 3**. Similarly, the LC−MS analysis showed six saponins with molecular masses of 1046, 1078, 1064, 1196, 1210, and 1224 Da in the KE preparation, and among them, saponin with a molecular mass of 1210 Da was the main one (ca. 36.0%) (**Table 1**). This study shows that this saponin has a glucose unit linked to position 26 with a sugar chain that consists of a glucose−glucose−rhamanose chain and xylose attached to position 3 and a methyl unit attached at position 22 of the diosgenin aglycon (*28*). Nine saponins with molecular masses of 1064, 1196, 1210, 1224, 1340, 1516, 1530, 1572, and 1586 Da with the main one at 1196 Da (ca. 52.3%) were found in the RE preparation (**Table 1**). This study shows that this saponin has a similar sugar chain and their attachment as 1210 Da saponin except in position 22, where it is attached at a OH group instead of a CH_3 group as in 1196 Da saponin (*28*). The MS/MS spectra of the main saponin of KE and RE are also presented in **Figure 2**. LC−MS analysis also showed that ME, KE, and RE saponin preparations used in this study make up 44.3, 35.5, and 39.1% of the total saponin amounts based on DW, respectively (**Table 2**). The *Q. saponaria* (QE) preparation is based on quilljic acid as the aglycon with glucuronic acid, galactose, and xylose sugar units attached at position 3 and fucose, apiose, xylose, arabinose, and rhamnose sugar unit attached at position 28 (*29*). The total sapogenin content of the QE preparation was at least 25% on the basis of the DW, according to the manufacturer's specifications. The spectrophotometric analysis showed 22−27% (DW basis) of total sapogenin to all *B. aegyptiaca* saponin preparation.

Effect of the Saponin Preparations on Penetration of 2,4-D across the CMs. The integrity of the isolated cuticles was tested 24 h prior to initiation of every experiment by adding a drop of

Table 2. Characterization of the *B. aegyptiaca* Saponin Preparations Used in This Study[a]

extract	total saponins (% DW)[b]	major saponins: molecular mass (Da)[c]	C26[d]	C3[d]	C22[d]
ME	44.3 ± 2.0	1064	Glu	Glu, Glu, Rha	OH
KE	35.5 ± 1.6	1210	Glu	Glu, Glu, Rha, Xyl	CH_3
RE	39.3 ± 2.4	1196	Glu	Glu, Glu, Rha, Xyl	OH

[a] ME, KE, and RE are the saponin preparations obtained from the fruit mesocarp, kernel, and roots of *B. aegyptiaca*, respectively. [b] Each value is the mean from three values ± the standard error, quantitatively derived via the ESI-MS-identified saponin peak area of the chromatograms obtained by HPLC-RI. [c] The molecular mass of the major saponin was determined by ESI-MS. [d] The molecule in the designated carbon is derived from the structure of each of the saponin extracts, and structure elucidation was carried out by high-field NMR (*28*). The structure of the major saponin is given in **Figure 2**.

Table 3. Effect of Saponin as an Adjuvant on the Rate of Penetration of [^{14}C]-2,4-D across the *C. grandis* Leaf Cuticular Membrane at 30 °C and 30% RH[a]

treatment	rate of penetration (h^{-1})	factor increase
DDW	0.59×10^{-5}d	0.0
RE	5.83×10^{-5}c	8.9
KE	6.18×10^{-5}c	9.5
QE	8.75×10^{-5}b	13.7
ME	10.20×10^{-5}a	16.3
Triton	11.00×10^{-5}a	17.6

[a] Together with 2,4-D, a 1% (w/v) solution of Triton, ME, RE, KE, or QE was added in each treatment as an adjuvant. Each value is the mean from the pool data of 60 CMs. Means sharing common postscripts are not significantly different ($P < 0.05$). Triton, QE, ME, KE, RE, and DDW refer to Triton X-100, *Q. saponaria* extract saponin, *B. aegyptiaca* fruit mesocarp extract saponin, *B. aegyptiaca* kernel extract saponin, *B. aegyptiaca* root extract saponin, and deionized distilled water, respectively. DDW contained only a 2,4-D solution as a control.

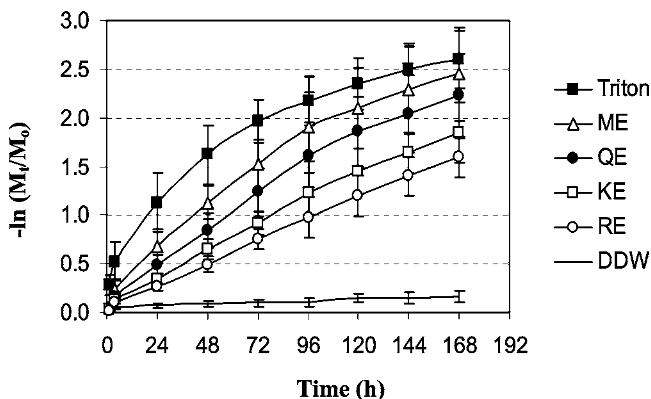

Figure 4. Time course effect of the different saponin preparations on penetration of [^{14}C]-2,4-D across *C. grandis* leaf cuticular membranes at 30 °C and 30% RH. Together with 2,4-D, a 1% (w/v) solution of Triton, ME, RE, KE, or QE was added in each treatment as an adjuvant. QE, ME, KE, RE, and DDW refer to the *Q. saponaria* preparation, *B. aegyptiaca* fruit mesocarp preparation, *B. aegyptiaca* kernel preparation, *B. aegyptiaca* root preparation, and deionized distilled water, respectively. The DDW contained only a 2,4-D solution as a control. Each value is the mean of 60 CMs ± the standard error.

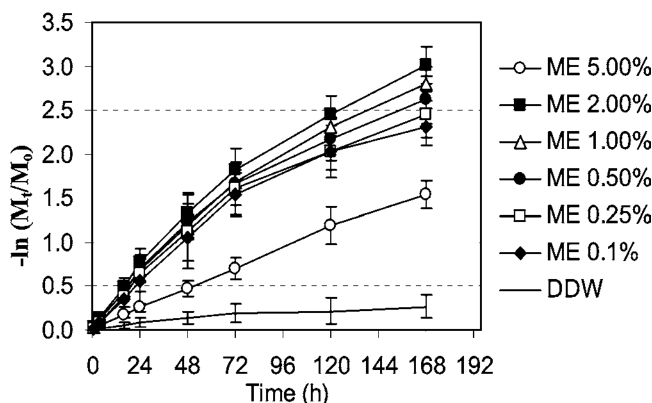

Figure 5. Time course effect of the concentration of the *B. aegyptiaca* fruit mesocarp preparation (ME) on the penetration of [^{14}C]-2,4-D across *C. grandis* leaf cuticular membranes at 30 °C and 30% RH. Each treatment contained the same concentration of 2,4-D together with the amount of ME as an adjuvant as listed on the graph. The DDW (deionized distilled water) contained only a 2,4-D solution as a control. Each value is the mean of 60 CMs ± the standard error.

20 μL of methanol in the center of each CM. Only CMs that showed no any leakage in the integrity test were used in the experiment. The integrity of the CMs was also confirmed by scanning electron microscopy (SEM) analysis of the inner and outer surfaces as shown in **Figure 1**. The effect of the different saponin preparations on the penetration of the [^{14}C]-2,4-D is presented in **Figure 4**. The rate of penetration of 2,4-D was highest initially, but it tended to level off with time. The first-order plot (natural logarithms) is the fraction of 2,4-D that had not yet penetrated versus time; thus, 2,4-D penetration can be completely described by a single constant, the rate constant (k) of penetration, which is equivalent to the slope of the straight line (*25*). When no adjuvant was added to the 2,4-D (control − DDW), the penetration rate was 0.59×10^{-5} h^{-1}. The penetration rate was 8.9 times higher (5.83×10^{-5} h^{-1}) when RE was used. Similarly, the rate was 9.5 times higher (6.18×10^{-5} h^{-1}) and 13.7-times higher (8.75×10^{-5} h^{-1}) with KE and QE treatment, respectively, whereas the rate was 16.3 times higher (10.20×10^{-5} h^{-1}) with ME treatment and 17.6 times higher (11.00×10^{-5} h^{-1}) with Triton X-100 treatment. Among all the saponin preparations, the highest penetration rate was achieved with ME, and this rate of penetration was not significantly different than that of Triton X-100 (a positive control) (**Table 3**).

Effect of *B. aegyptiaca* ME Concentration on the Penetration of 2,4-D across CMs. The first phase of the study showed that the commercial synthetic foliar adjuvant Triton X-100 and ME preparation significantly enhanced the penetration of 2,4-D across the CMs in comparison to the control and other saponin preparations. Since there was no significant difference in the rate of penetration between ME (1%) and Triton X-100, in a second phase of the study a series of concentrations of ME were tested. In this study, ME concentrations were used in a range from 0.1 to 5.0% (**Figure 5**). The rate of penetration of the control treatment (without any addition of an adjuvant, only 2,4-D called here DDW) was just 0.59×10^{-5} h^{-1}, which was almost 19 times lower than the value for 2% ME, which had the highest penetration rate among all treatments. However, the rate of penetration for 1% ME was not significantly different from that for 2% ME (**Table 4**). Rates of penetration increased with an increase in ME concentration from 0.1 to 2.0%; however, the rate of penetration was drastically decreased when 5% ME was used (5.65×10^{-5} h^{-1}) in comparison to 2% ME (11.90×10^{-5} h^{-1}).

Effect of Humidity and Temperature on the Penetration of 2,4-D across CMs. In the third phase of the study, the effects of humidity and temperature on the rate of penetration of 1%

Table 4. Effect of the Concentration of the *B. aegyptiaca* Fruit Mesocarp Saponin Extract (ME) as an Adjuvant on the Rate of Penetration of [14C]-2,4-D Across the *C. grandis* Leaf Cuticular Membrane (CMs) at 30 °C and 30% RH[a]

treatment	rate of penetration (h^{-1})	factor increase
DDW	0.59×10^{-5}f	0.0
0.10% ME	9.46×10^{-5}c	15.0
0.25% ME	9.88×10^{-5}bc	15.7
0.5% ME	10.53×10^{-5}b	16.8
1.0% ME	11.07×10^{-5}a	17.7
2.0% ME	11.90×10^{-5}a	19.1
5.0% ME	5.65×10^{-5}e	8.5

[a] Each treatment contained the same concentration of 2,4-D together with the reported amount of ME as listed. Each value is the mean from a data pool of 60 CMs. Means sharing common postscripts are not significantly different ($P < 0.05$). DDW refers to deionized distilled water used as a control.

Table 5. Effect of Humidity and Temperature on the Rate of Penetration of [14C]-2,4-D across the Astomatous Adaxial *C. grandis* Leaf CM with 1% *B. aegyptiaca* Fruit Mesocarp Extract Saponin (ME) as an Adjuvant[a]

	rate of penetration (h^{-1})
humidity[b] (%)	
30	10.46×10^{-5} (0.71×10^{-5})
60	13.32×10^{-5} (2.45×10^{-5})
90	16.35×10^{-5} (4.36×10^{-5})
temperature[c] (°C)	
30	10.35×10^{-5} (0.59×10^{-5})
60	12.32×10^{-5} (2.44×10^{-5})
90	16.15×10^{-5} (4.76×10^{-5})

[a] Values in parentheses are the rates of penetration of the control in the respective treatment under the respective conditions. [b] The humidity experiment was conducted at 30 °C. [c] The temperature experiment was conducted at 30% humidity.

ME across the CMs were tested. When humidity was increased from 30 to 60% and then to 90%, the rate of penetration of 2,4-D was found to increase from 10.46×10^{-5} to 13.32×10^{-5} h^{-1} and then to 16.35×10^{-5} h^{-1}, respectively. At all three tested humidity levels, the rate of 2,4-D penetration was dramatically increased in comparison to the control (**Table 5**). As with humidity, the rate of 2,4-D penetration also increased with an increase in temperature from 30 to 60 °C and then to 90 °C (10.35×10^{-5}, 12.32×10^{-5}, and 16.15×10^{-5} h^{-1}, respectively).

Electron Microscopy and Particle Size Characterization of Saponin Preparations. When all four saponin preparation solutions (QE, ME, KE, and RE) that were used earlier in different 2,4-D delivery experiments were characterized in TEM, small nanosized vesicles were clearly observed in all four solutions (**Figure 6**). Using dynamic light scattering measurements, the average diameter of the mean mass vesicle population of the ME, KE, RE, and QE solutions (1.0%) was obtained (**Figure 7**). The mean diameters of the ME and QE solutions were 167 and 177 nm, respectively, whereas the average diameters of the vesicles of the mass population of KE and RE were almost 3 times higher than those of the two previous saponin preparations, i.e., 502 and 587 nm, respectively. The data collected by these two systems suggest that the size and structure of the vesicles in both QE and ME were similar, whereas both the average size and structure of RE and KE were slightly different from those of QE and ME. In QE and ME, the nanosized vesicles were small and tightly spherical, whereas large and loose spherically shaped structures were observed in both KE and RE.

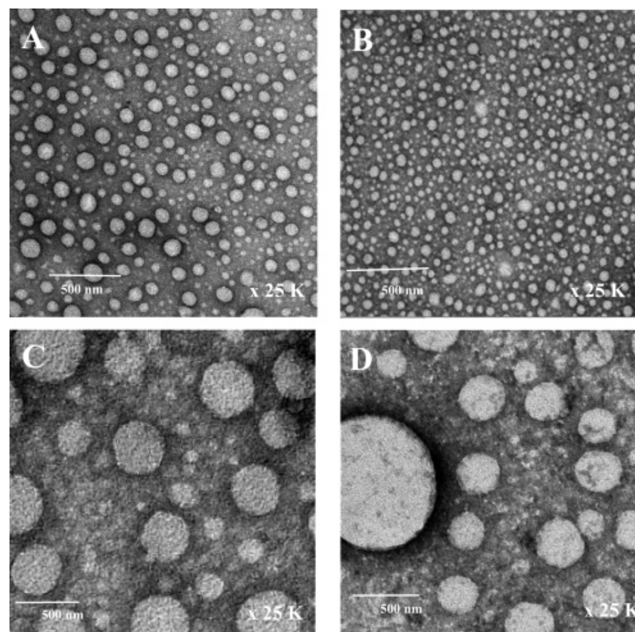

Figure 6. Transmission electron microscope (TEM) characterization of the nanovesicles present in the different saponin preparation solutions (1.0%): (**A**) *Q. saponaria* preparation (QE), (**B**) *B. aegyptiaca* fruit mesocarp preparation (ME), (**C**) *B. aegyptiaca* kernel preparation (KE), and (**D**) *B. aegyptiaca* root preparation (RE) with uranyl acetate background as negative staining at 25000× magnification.

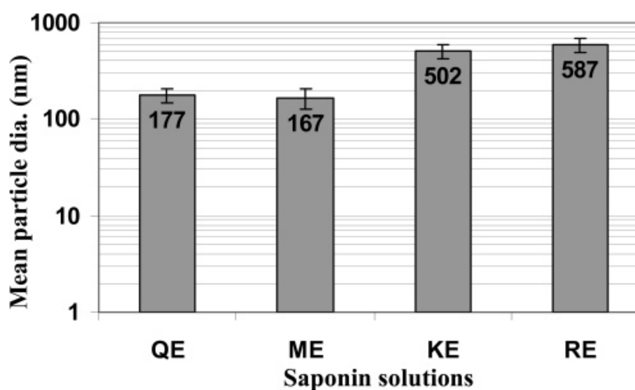

Figure 7. Mean vesicles size of the different saponin preparation solutions (1.0%) determined by the light scattering measurements (ALV-NIBS high-performance particle sizer) at a 173° angle at 632 nm (λ) and 25 °C. QE, ME, KE, RE, and DDW refer to the *Q. saponaria* preparation, *B. aegyptiaca* fruit mesocarp preparation, *B. aegyptiaca* kernel preparation, *B. aegyptiaca* root preparation, and deionized distilled water, respectively. Each value is the mean of six samples ± the standard error.

DISCUSSION

Many variables, both external and internal, regulate the rates of absorption of applied materials by plant foliage. Humidity, temperature, and the chemical constituents of the agromaterials are the main factors that influence foliar penetration of active materials into a plant (*30*, *31*). Since the cuticular membrane is composed of a lipophilic layer, the nature of applied materials (hydrophilic or lipophilic) affects the penetration. That is why lipophilic surfactants are commonly used to help penetration. The surfactants lower the surface tension and consequently increase the permeability and absorbance of applied materials.

High humidity and moisture on external surfaces, and low-moisture tension within the plant, favor rapid foliar uptake (*32*, *33*).

This study was an attempt to develop a natural phytoadjuvant system for delivery of agromaterials through the plant cuticle membrane. We observed a significant ability of a natural phyto-saponin preparation to deliver 2,4-D through isolated leaf CMs.

The first phase of the delivery experiment showed that saponin preparation from *Q. saponaria* bark and various parts of *B. aegyptiaca* accelerated the rate of penetration of 2,4-D through the astomatous adaxial *C. grandis* leaf CMs. One of the most common and leading synthetic surfactants used in foliar application, Titron X-100, showed the highest rate of 2,4-D penetration; however, this was not significantly different from that of the saponin preparation of *B. aegyptiaca* fruit mesocarp (ME).

In the second phase of the study, when a series of concentrations of ME was used, 1 and 2% ME optimized the rate of penetration of 2,4-D. Increasing the concentration to 5% inhibited the rate of penetration, which might be explained by crystallization of saponin molecules in the oversaturated solution (*8*, *34*). This suggestion is supported by scanning electron microscope (SEM) characterization of the interaction of saponin concentration with CMs (data not shown).

Increasing the relative humidity to 90% markedly accelerated the rates of penetration of 2,4-D through isolated CMs. Similarly, increasing the temperature from 30 to 90 °C markedly increased the rate of penetration from 10.35×10^{-5} to 16.15×10^{-5} h^{-1} (**Table 5**). These results are in good agreement with literature reports with regard to surfactant behavior (*8*, *25*).

Different possible reasons might be suggested for the acceleration of the rate of penetration of 2,4-D with the help of phyto-saponins as a delivery adjuvant. The first possibility is the surfactant effect of the saponin preparation on the cuticle layer that accelerates the diffusion process. It is common to use surface-active agents or surfactants as spray adjuvants in formulations of agrochemicals to improve their effectiveness following application to foliage (*7*). Saponin compounds function as surface-active agents because of the lipophilic and hydrophilic moieties present in the molecule. The structure of the saponin preparations used in this study (**Table 2** and **Figure 3**) has clearly demonstrated the presence an amphipathic structure with both lipophilic (aglycone) and hydrophilic (sugar chains) portions in line with the literature report as common natural surfactants (*15*). Saponins, which are steroidal and triterpenoid glycosides, have the ability to lower the surface tension of aqueous solutions (*35*). The glycosidic saponins as natural surfactants keep the delivered biomaterial in a solubilized or partially solubilized form, depending mainly on the environmental humidity and temperature conditions (*25*), and they may increase the plasticity and permeability of biological membranes (*8*).

The second possibility is the phenomenon of binding of saponin compounds to phytosterols that are integrated in leaf CMs (*3*, *4*). Although the precise mechanism of how saponins interact with the membrane is not fully understood (*32*), it is generally accepted that the mechanism of their involvement in a wide spectrum of biological processes is closely related to their high affinity for cholesterol structures that are stabilizing biological membranes (*13*). This interaction is described well with regard to the antifungal activity of saponins. In this case, it was clearly demonstrated that because of their ability to form a complex with sterols in fungal cuticle membranes, saponins cause a loss of membrane integrity (*32*). It has further been reported that saponins form a complex with sterols in membranes and ultimately form aggregates which then lead to the formation of membrane pores (*34*). Hence, one of the possibilities behind the penetration could be the affinity of glycosidic saponin compounds from saponin preparation for improved association with CMs and their affinity for the sterol compounds of these membranes. Therefore, there is a good chance that these saponins cause significant disorders in the cuticles. The data obtained in the 2,4-D delivery system clearly demonstrated the interaction between saponin preparations and CMs. These data extend and support the previous data on interaction of saponins with biological membranes (*15*). This may also contribute to the understanding of the interference of saponins with a wide range of biological systems.

The third possible reason behind the penetration rate enhancement is the formation of nanovesicles of the micelle type that may help in the delivery of 2,4-D. Microscopic study of the saponin preparation used in our studies has shown the formation of self-assembled nanosized vesicles (**Figures 6** and **7**). Various nanoscale vesicle systems, including micelles, have been suggested and demonstrated to be vehicles for the delivery of different compounds (*13*). A previous report characterized the *Q. saponaria* saponin micelle assembly and related it to the critical micelle concentration (CMC) (*14*, *29*). In agreement with these reports, we observed similar behavior for the *B. aegyptiaca* saponin preparation and the *Q. saponaria* preparation. Vesicle formation, which is exclusively characteristic of amphiphatic compounds, further supports the dominance of saponin compounds in these preparations and rules out the possibility that the adjuvant effect on 2,4-D delivery may come from other compounds extracted from the plant tissues used in this study. The vesicle size measured for ME and QE preparations (<200 nm for both) may also contribute to the explanation of the increased rate of 2,4-D penetration through CM by these two preparations in comparison to those of the other two preparations (KE and RE, which formed >500 nm vesicles). The increased rate of penetration due to the increasing concentration of the ME preparation ($\leq 2.0\%$) may be explained by the lyotropic response reported for glycolipid saponin compounds self-organized in vesicle form (*37*). A higher level of relative humidity is well-known to accelerate the rate of penetration by increasing the diffusion driving force (*8*, *25*, *33*). The effect of increased temperature on the rate of 2,4-D delivery may be explained by an increase in CM plasticity and also may be related to the thermotropic response of the saponin compounds in an aqueous medium (*37*).

Another aspect that may contribute to the understanding of the different rates of penetration of 2,4-D across the leaf cuticle membrane using ME, KE, and RE could be related to the differences in saponin constituents in these preparations. Although ME, KE, and RE all are saponin preparations obtained from the same plant, *B. aegyptiaca*, chemical characterization of the saponins produced in different tissues of this plant shows that there are compositional differences of saponins in *Balanites* plant tissues (**Table 2** and **Figure 3**). The characterization of the different *Balanites* tissues for saponin constituents shows that the major saponin in fruit mesocarp contains one fewer pentose sugar unit (xylose) than the root and kernel saponins. Furthermore, there is also a difference in the presence of a CH_3 and OH group in position 22 in the major saponin prepatration of *Balanites* kernel and root tissues, respectively (**Figure 3**). As suggested by Goodby et al. (*37*), these structural differences may affect the self-organization of the saponin compounds in the aqueous solution and further influence the different delivery

results obtained in this study. Along those lines, some additional studies carried out by us, behind the frame of the research presented here, showed that biological activities of saponin preparations from mesocarop, kernel, and root tissues significanly vary (*18*), and these variations may also be attributed to the saponin structural differences.

From the results realized in this study, it is clearly shown that saponin preparations of both *Q. saponaria* and *B. aegyptiaca* accelerate the delivery of 2,4-D through isolated leaf cuticle membranes. Hence, the results indicate that amphiphilic saponins could be used as phytoadjuvants for the delivery of agrochemicals through CMs. *Q. saponaria* has already been exploited for its commercial uses; however, the use of *Balanites* is very negligible. Since *Balanites* is highly adapted to most of the arid land where other crops are extremely difficult to grow (*19*), the product of *B. aegyptiaca* could be very inexpensive. Furthermore, *B. aegyptiaca* contains a large quantity of easily extractable saponins in its fruit mesocarp, which is not popular for fresh fruit consumption. Thus, the results of this study also open the door for the development of this neglected desert plant species, *Balanites*, as a valuable product. Since saponins are environmentally friendly, natural substances, the plant-originated natural saponin-based delivery system could play a vital role in saving the environment from pollution. The antifungal and antimicrobial properties of these saponins would help judicious placement of agrochemicals in the framework of integrated pest management, as well.

ABBREVIATIONS USED

2,4-D, 2,4-dichlorophenoxyacetic acid; CMs, astomatous adaxial *C. grandis* leaf cuticle membranes; CMC, critical micelle concentration; DDW, deionized distilled water; DW, dry weight; Glu, glucose; M_0, amount applied; M_t, amount penetrated; KE, kernel saponin extract of *B. aegyptiaca*; ME, mesocarp saponin extract of *B. aegyptiaca*; QE, bark saponin extracts of *Q. saponaria*; RE, root saponin extract of *Balanites* saponins; Rha, rhamnose; SC, stratum corneum; SEM, scanning electron microscope; TEM, transmission electron microscope; Xyl, xylose.

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